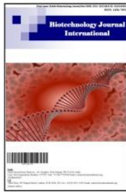

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Construction of an Agrobacterium Mediated RNAI Genetic Transformation Vector Targeting the Replicase Gene of *Indian cassava mosaic virus* and Evaluation of Their Transformation Ability in Cassava Immature Leaf Lobes

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Authors' contributions

This work was carried out in collaboration between all authors. Author TA designed the study, performed the experiments, wrote the protocol and wrote the first draft of the manuscript. Author PP managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Cassava mosaic virus is one of the major problems affecting cassava industry in India. Currently there are no effective strategy to completely protect cassava from cassava mosaic viruses. In order to attain cassava mosaic virus resistance RNAi vectors targeting the replicase gene of Indian cassava mosaic virus is constructed in this study. Their efficiency to transform cassava immature leaf lobes were also studied here. Replicase gene of Indian cassava mosaic virus in Tamilnadu are cloned and sequenced. Conserved domains are identified and sub cloned to CSIRO RNAi vector system and transformation studies are done in immature cassava leaves. Two different RNAi vectors were constructed, utilizing a conserved 440bp of 5' end of ICMV Rep (AC1) gene which also corresponds to a part of AC4 gene, and functions as a viral RNAi suppressor protein. The partial *Rep* gene of ICMV was cloned in sense and anti-sense orientations in the RNAi intermediate vector, pHANNIBAL. After cloning into pHANNIBAL, the cloned RNAi gene cassettes of ICMV is released and cloned into the binary vector, pART27, which contains the kanamycin-resistance

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gene as a plant selectable marker. In order to use hygromycin as a selection agent in cassava genetic transformation, RNAi–Rep gene cassettes of ICMV were cloned into pCAMBIA1305.2. These constructs were named pICR1 and pICR2 respectively. The Genetic transformation studies in cassava leaves done using pICR2 vector could generate PCR positive plants. An agrobacterium mediated replicase RNAi vector is developed and that can be transformed into cassava immature leaf lobes. Their efficiency to silence the Indian cassava mosaic virus should be studied further.

Keywords: RNAi constructs; Indian cassava mosaic virus; replicase, binary vector; genetic transformation.

1. INTRODUCTION

Cassava (*Manihot esculenta*) is a major tuber crop cultivated in 13 states of India. Cassava is grown in an area of 2.34 mha in India, with Kerala ranking first in area (1.04 mha) followed by Tamil Nadu (0.95 mha) [1].Cassava production is affected by a combination of biotic and abiotic stresses, among them; cassava mosaic disease (CMD) caused by Cassava Mosaic Gemini viruses (CMGs) limit the productivity of cassava. CMD had not been reported in India before 1966 and has become more prevalent in recent years in Southern India especially Salem, Dharmapuri districts in Tamilnadu and almost all parts of Kerala and causes severe yield loss ranging from 25-80%. The main reason for the fast spread of the disease is due to the indiscriminate use of the infected planting material. Most of the popular varieties grown in Tamil Nadu are either susceptible (includes H-226, Sree Harsha) or tolerant (includes H165, Co-1, Co-2 and MDV2) to the disease. None of the varieties grown in Tamil Nadu are resistant to the disease. Conventional breeding is not successful in developing resistant varieties, which is seriously limited due to long growth cycle, highly heterozygous in nature, poor seed set and viability.

Cassava Mosaic Disease (CMD) in India is caused by two species of Begomoviruses viz., Indian Cassava Mosaic Virus (ICMV) and Sri Lankan Cassava Mosaic Virus (SLCMV). Both the DNA viruses belonging to the bipartite Begomovirus genus of the family Geminiviridae, and are transmitted by whitefly *Bemisia tabaci* Genn. Transformation and regeneration of transgenic cassava plants expressing kanamycin resistant gene as selectable marker has been reported by several laboratories [2,3,4,5,6].

Several reports have shown that RNA interference (RNAi) is more potent in controlling plant virus than the sense or antisense expression of the viral genes. It is now well established that both RNA and DNA viruses can be controlled by RNAi approach. The RNA viruses are effectively controlled by silencing the coat protein gene, whereas, the DNA viruses are effectively controlled by silencing the *Rep* gene, which is indispensable for DNA replication of virus Pooggin et al. [7]. As a proof of the concept that RNAi can be engineered to effectively target DNA virus namely, Mung Bean Yellow Mosaic Virus (MYMV-Vig) was demonstrated by Pooggin et al. [7].The DNA-A of the CMV codes for the AC1 gene, the replication-associated protein gene (or *Rep* gene), which is indispensable for the replication of virus and disease development. In this study, an attempt was done to identify the conserved domain of replicase gene of Indian cassava mosaic virus through gene cloning and clustal analysis, which can be used to target gene silencing. Binary vectors are constructed and tested for their efficiency to transform immature leaf lobes of cassava.

2. MATERIALS AND METHODS

2.1 Isolation and Characterization of ICMV Rep Gene

All the studies in this research were done at Department of Plant biotechnology and molecular biology, Tamil nadu Agricultural university, Coimbatore. The virus infected cassava leaf samples (A total of 44 symptomatic leaf samples) were collected from different cassava growing areas of Tamilnadu like Salem, Coimbatore, Dharmapuri, Attur, Namakkal and one sample from Kerala.

2.1.1 Designing of gene specific primers

The complete nucleotide sequence of Rep gene of ICMV (Accession No. AJ314739.1,AY730035.2,Z24758.1,AJ575819.1, AY149901.1,DQ658178.1,AY738105.1) were downloaded from NCBI Genebank. The ICMV Rep gene sequences were multiple aligned separated using ClustalX programme and conserved regions were found

2.1.2 PCR analysis of rep gene

The DNA extracted(C-TAB method) from infected cassava leavesfrom 5 districts of Tamilnadu was used for PCR amplification of viral *Rep* gene. Reactions were performed in a final volume of 20 µl and the mixture contained 2 µl (100 ng) of diluted total genomic DNA, 4.0 µl of 5X GC Phusion PCR buffer, 0.4 µl of 10 mM dNTPs, 0.5 µl of 0.5µM of respective forward and reverse primers, 0.2 µl of 2 U Phusion™ hot start high fidelityDNA polymerase (Finnzyme, Finland) and 13.9 µl sterilized double distilled water. Temperature profile and PCR cycles used for gene specific primerwere94ºC for 5 min - Initial Denaturation, 94ºC for 1 min - Denaturation, 58ºC for1.0 min - Primer annealing (30 cycles), 72ºC for 2 min - Primer extension and 72ºC for 15 min - final extension. Amplified PCR products were electrophoresed through 1 % (w/v) agarose gel.

2.1.3 Ligation and transformation

The ligation reaction for blunt end cloning was set up in a final volume of 20 µlwith 10 µl of 2X reaction buffer, 1-2 µl of PCR product (non purified), 1 µl of 50 ng pJET1.2/blunt cloning vector, 1 µl of (5 u/µl) T4 DNA ligase (CloneJet PCR cloning kit; Fermentas) and Nuclease free water was added to make the total volume upto 20 µl. The ligation mixture was vortexed briefly for 4-5 seconds and incubated at 22º C for 5 min. Two microliters of the ligation mixture was directly used for bacterial transformation. To an aliquot of 50 µl of DH5α competent cells, 2.5 µl of ligation mixture was added. The mixture was incubated on ice for 30 min and a heat shock was given at 42°C using a water bath for 90s and again incubated on ice for 5-10 min. Then the contents were transferred to 1 ml LB broth and incubated at 37°C with 200 rpm shaking for 1h. After incubation, 100 µl of the cell suspension was uniformly spread on LB agar (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract, 15 g/l agar, pH 7.2) medium containing ampicillin (100 mg/l) and incubated at 37°C for overnight, for the colonies to develop. All recombinant colonies will appear on the plate.

2.2 Sequencing and Phylogenic Analysis of Cloned Isolates of ICMV and SLCMV

The recombinant colonies were sent for sequencing (Genei Bangalore). The sequencing result was analysed through NCBI BLAST search. The BLAST programme was used to identify related sequences available from the Genbank databases as well as to get the homology percentage. The conserved sequences were found using Bio Edit to design primers.

2.3 Construction of Gene Silencing Vectors with *npt* and *hpt* Selectable Marker Gene

The hair pin binary vectors are made using CSIRO RNAi vector construction steps using pKANNIBAL vector. A conserved sequence of size 440 bp from the 5' region of the Rep gene sequence ofICMV were identified and primers were designed to amplify 440 bp Rep gene of ICMV covering 112 – 540 nt region of Rep gene. The restriction enzymes, *Xho*I and *Kpn*I were appended with sense forward and reverse primer and *BamH*I and *Hind*III were appended with antisense forward and reverse primer ofICMV respectively. The PCR product is then cloned sequentially on either side of the pdk intron to become the two arms of the hairpin. The complete hairpin RNAi gene cassette was released by digestion with *Not*I restriction enzyme and cloned into pART27, plant transformation binary vector from CSIRO Plant Industry, Australia. The constructed pART27 vectors with RNAi-ICMV Rep cassette was designated as pICR1.Also the RNAi cassette of ICMV was further cloned into the *Not*I site of pBLUESCRIPT (SK+) vector and then the insert was released with *Sal*I and *Sac*I restriction digestion and cloned into pCAMBIA1305.2 which has hpt selectable marker gene. The recombinant clones were identified by restriction analysis with *Sal*I and *Sac*I enzyme. The recombinant plasmids were named pICR2.

3. RESULTS

3.1 Isolation and characterization of Rep Gene of ICMV from Tamil Nadu

Using the designed gene specific primers, the expected full length ICMV Rep gene of size 1058 bp were obtained by PCR amplification in all the symptomatic leaves. The PCR amplified products were then cloned into pJET1.2/Blunt cloning vector, (Fermentas, USA). The colony PCR was done with gene specific as well as pJET vector specific primers amplified the expected amplicon size of 1058 bp for ICMV from the recombinant colonies of ICMV. In case of recombinant clones with ICMV Rep gene, the clones were further confirmed by restriction digestion with *Bgl*II to release the 1058 bp DNA fragment. DNA

sequencing confirmed the cloning of Rep gene of ICMV as identified by NCBI blast search. The forward and reverse DNA sequence of each clones were aligned to get the complete DNA sequence of each clone. Then the DNA sequences were submitted to NCBI Gene Bank, USA and temporary Accession numbers were assigned for the ICMV by Gene Bank.

3.2 Construction of RNAi-Rep Vector for Control of ICMV

A partial 440 bp Rep gene (112- 540 nt region of Rep gene) of ICMV was selected as the target region for gene silencing and was amplified using designed primers. The selected region also corresponds to a part of the AC4 gene, which lies within the AC1 gene and function as a suppressor protein in gene silencing. This partial Rep gene was first cloned into Sense orientation in pHANNIBAL vector in the *Xho*I/*Kpn*I site. The recombinant clones were confirmed by colony PCR using Rep gene specific forward and reverse primer which amplified an expected size of 440 bp. Then the pHANNIBAL cloned with sense Rep gene was used for cloning the antisense Rep gene in the *Hind*III/*Bam*HI site. The recombinant clones after cloning antisense of Rep gene was identified by colony PCR with reverse primers of Rep gene and Ocs terminator, which amplified an expected amplicon of 540 bp. The recombinant clones containing both the sense and antisene of the Rep gene were further confirmed by *Xho*I /*Hin*dIII and *Kpn*I/*Bam*HI double digestion, which released the 1.2 kbp fragmen.t The 1.2 kbp fragment includes the 760 bp of pdk intron and 440 bp of Rep sense and Antisense gene. The 3.8 kbp RNAi –ICMV Rep gene cassette was released by *Not*I restriction digestion.

3.3 Cloning of Rep-RNAi Cassettes in pART27 Vector

The released 3.8 kb RNAi Rep gene cassette fragment from pHANNIBAL was cloned into the *Not*I site of the plant transformation vector, pART27 (CSIRO, Australia). The presence of the insert in the pART27 vector was confirmed by colony PCR using the anti sense Rep gene primer and Ocs terminator primers. After identifying the recombinant clones in colony PCR, this was further confirmed by restriction digestion with *Not*I as well as with *BamH*I and *Sal*I . The *Not*I digestion released two fragments; the 3.8 kbp RNAi-Rep gene cassette and the vector backbone of 11.6 kb. The double digestion with *BamH*I and *Sal*I will cut the plasmid at three sites and released fragments of sizes 8.1 kbp, 3.8 kbp, 2.3 kbp and 1.18 kbp. The recombinant plasmid vector was designated as pICR1. With a view to characterize the RNAi gene cassette after cloning into pART27, different primers were designed to obtain the complete DNA sequence of the gene cassette. The DNA sequence of complete RNAi-Rep gene cassette of pICR1 vector was done. The binary vectors pICR1 was mobilized into the *Agrobacterium* strain LBA4404 by Triparental mating method.

3.4 Cloning of Rep-RNAi Cassettes in pCAMBIA Vectors

In order to use hygromycin as selection agent in cassava genetic transformation, Rep-RNAi gene cassettes of ICMV was cloned into pCAMBIA1305.2 vector that contain the *hph* gene and Gus gene. Since *Not*I restriction site is not available in the pCAMBIA vector, the RNAi gene cassettes were first sub cloned into *Not*I site of pBLUESCRIPT (SK+) vector. Then the 3.8 kbp RNAi gene cassette was released from the pBLUESCRIPT by double digestion with *Sal*I/*Sac*I. The 3.8 kb Rep-RNAi fragment was then cloned into *Sal*I and *Sac*I site of pCAMBIA 1305.2. The recombinant clones containing the Rep-RNAi cassette in pCAMBIA 1305.2 was confirmed by double digestion with *Sal*I and *Sal to release the* 3.8 kb cassette .The RNAi binary vectors of ICMV with this pCAMBIA1305.2 were named pICR2. A preliminary genetic transformation of cassava explants was done using pICR2 binary vectors.

4. DISCUSSION

Cassava (*Manihot esculenta)* is a staple food for 600 million people in the tropical and subtropical belt, as well as a feedstock for numerous industrial applications, including food, feed and starch. Cassava production in India is seriously hampered by the occurrence of two strains of cassava mosaic virus, ICMV and SLCMV leading to a serious decline of the crop and drastic yield reduction.

ICMV and SLCMV are Geminiviruses characterized by small geminate particles (18-20 nm) containing two single-stranded circular DNA molecules, DNA-A and DNA-B of ~2.7 kb [8]. DNA-A has six genes: AC1 encodes a replication-associated protein (Rep) essential for viral DNA replication in association with host DNA polymerase [9]; AC2 encodes a

transcription activator protein (TrAP) [10]; which also found to have PTGS suppression role in ICMV [11]; AC3 encodes a replication enhancer protein (REn) [12]; AC4 function as PTGS suppressor protein [11]. AV1 and AV2 encode coat protein and pre-coat protein, respectively [13]; and DNA-B has BV1 and BC1 genes that encode a nuclear-shuttle protein (NSP) and movement protein (MP), respectively.

Screening the cassava germplasm for natural resistance and conventional breeding were some of the initial attempt to obtain CMD resistance. However, most of the popular elite cultivars grown in India are either susceptible (includes H-226, Sree Harsha) or moderately tolerant (includes H-165, Co-1, Co-2 and MVD2) to CMV. The high heterozygosity and inbreeding depression complicate conventional breeding in cassava. However, to enhance the efficiency of cassava breeding a number of resources and molecular tools have been developed during the recent years. This include the construction of genetic maps using RFLP, isoenzymes, microsatellite markers [14,15] that have already allowed the identification of a variety of QTLs and a major gene (CMD2) for CMD resistance. However, such markers are limited in their application to breeding, and a more precise approach to gene mapping using candidate genes is required.

The number of identified and studied Geminivirus related R genes are very less and these genes are yet to be cloned and used for cassava genetic improvement. The lack of availability of natural resistant genes in the germplasm makes the pathogen derived resistance as one of the useful method for achieving viral resistance in cassava. This concept was first postulated by Hamilton in 1980 [16].Currently, there are two basic molecular mechanisms by which PDR is thought to operate, protein mediated resistance in which the expression of an unmodified or modified viral gene product (includes genes for coat protein, movement protein and replicase protein) interferes with the viral infection cycle and secondly, RNA mediated resistance, which does not involve the expression of a protein product. The RNA based resistance mainly includes antisense RNA technology and RNA interference. RNA interference (in plants Post Transcriptional Gene Silencing) describes one of the powerful innovations which can be directly applied to evolve crops resistant to stress caused by virus [7].

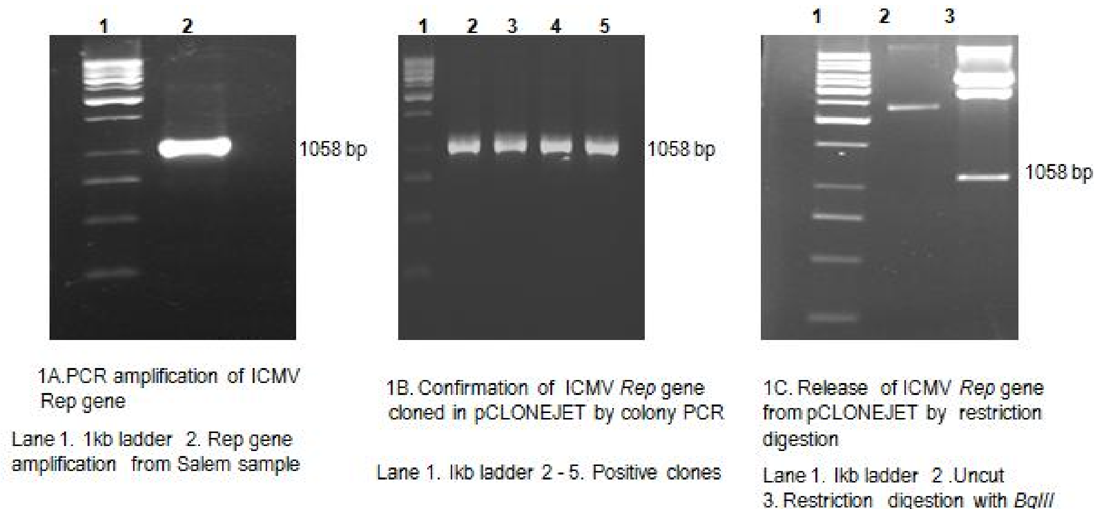
Two research institutes, the Danforth Plant Science Center (DPSC) in the USA and ETH-Zurich, Switzerland, have been engaged in development of transgenics in cassava, with both groups employing pathogen-derived resistance (PDR) strategies. Successful generation of transgenic cassava resistant to ACMV was reported through expression of full length ACMV Rep gene at Donald Danforth Center, USA [11]. At ETH the approaches for the control of Gemini virus include the expression of antisense viral sequences which interrupt the virus proliferation at the level of DNA replication [17,5]. To overcome CMV disease problem, genetic engineering approach provides scope for imparting CMD resistance in Cassava.

Hence, in the present study, RNAi approach which targets the Rep gene of ICMV was followed to engineer resistance in cassava. Rep gene is very essential for the Geminivirus viral DNA replication. The Gemini virus multiplication relies mostly on the host DNA replication apparatus with only one virus-encoded protein replication initiator protein (Rep). The single-stranded DNA genome of the virus is replicated in the host nuclei via double-stranded DNA intermediates using a rolling circle mechanism [18]. The product of the AC1 ORF, Rep, plays its key role by initiating the rolling circle replication by virtue of its nicking and ligation property. An entire ORF AC4 gene lies within the 5' end of the AC1 gene is characterized as a pathogenicity determinant as well as a suppressor of RNAi [19]. Hence, the region of AC1 gene with the overlapping AC4 gene was taken for RNAi vector construction to get an effective long lasting resistance towards CMD.

In addition, Rep gene was chosen as targets in this study for two reasons. First, it is well known that Rep mediated resistance is successful against Geminiviruses. Furthermore, it has been already reported that the transient expression of full length AC1 gene of ACMV [11] or the truncated N-terminal portion of the Rep protein or a mutated Rep gene in the NTP binding domain caused a significant reduction in the level of viral DNA replication in *Nicotiana tabacum* protoplasts [20].

To achieve the RNAi mediated resistance in cassava, a 440 bp region of ICMV Rep gene which overlap with the AC4 gene was used for vector construction. Wesley et al. [21] reported that use of 98 nt to 853 nt size gene target gave efficient silencing in a wide range of plant

species. This 440 bp region was first cloned in sense and antisense orientation in an RNAi intermediate vector, pHANNIBAL containing a splicable intron, pdk. It is reported that linking the sense and anti-sense sequences by an intron, which is eventually spliced, resulted in the most efficient silencing in plants [22,18]. It is well documented that intron containing constructs (ihpRNA) generally gave 90±100% of independent transgenic plants showing silencing. The degree of silencing with ihpRNA constructs was much greater than that obtained using either co-suppression or anti-sense constructs. This RNAi hairpin vectors (designated as pICR1 and pICR2) targeting the Rep gene of ICMV were transformed into Cassava variety H-226.



1A.PCR amplification of ICMV Rep gene

Lane 1. 1kb ladder 2. Rep gene amplification from Salem sample

1B. Confirmation of ICMV *Rep* gene cloned in pCLONEJET by colony PCR

Lane 1. Ikb ladder 2 - 5. Positive clones

1C. Release of ICMV *Rep* gene from pCLONEJET by restriction digestion

Lane 1. Ikb ladder 2 .Uncut
3. Restriction digestion with *BglII*

Fig. 1. Cloning of ICMV Rep gene from symptomatic leaves of cassava

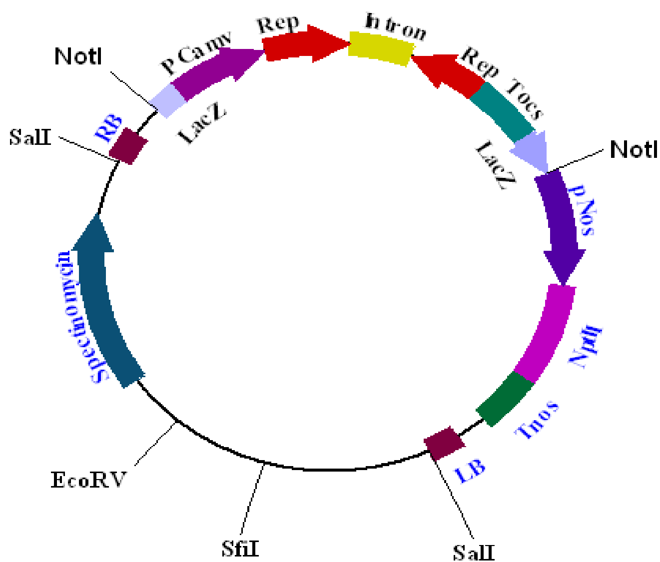


Fig. 2. pART27 with ICMV replicase hairpin construct

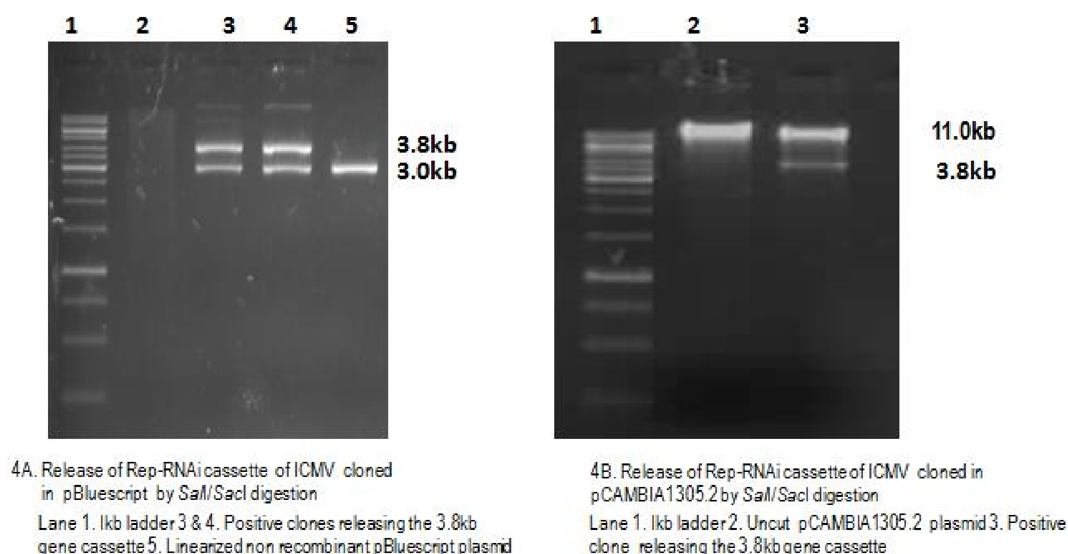


Fig 3. Constructions of Rep-RNAi construct of ICMV in pCAMBIA 1305.2 binary vector

5. CONCLUSION

The present study developed a binary vector targeting the replicase gene of Indian cassava mosaic virus and it was transformed into cassava immature leaf lobes. This is a stepping stone towards genetic engineering cassava for generating cassava mosaic virus resistance through RNA interference. Transgenic cassava plants developed should be further assesses for virus resistance and si RNA accumulation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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