DEVELOPMENT OF TRANSGENIC CASSAVA WITH REPLICASE GENE (AC1) OF INDIAN CASSAVA MOSAIC VIRUS

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Indian Cassava Mosaic Disease

Field view

Indian Cassava Mosaic Disease

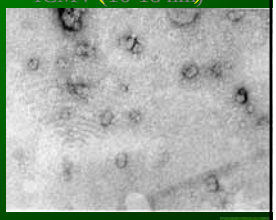
Mosaic

Leaf Distortion

Cassava Mosaic Disease

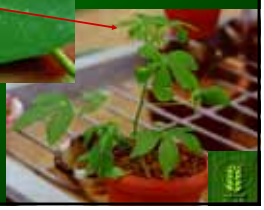
Severe Symptom

ICMV (16-18 nm)



Cassava Mosaic Disease

ICMV transmission through whitefly



Field level spread through whitefly is up to 10%

Importance of CMD in India

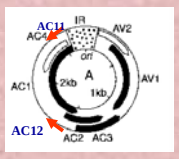
- Occurs in more severe form in Kerala and Tamil Nadu
- Emerging as a problem in other states
- It causes yield loss ranging from 25 – 80%
- No cultivar is resistant to this disease
- Factors:
  - Indiscriminate use of infected planting material
  - Non adoption of rogueing / clean cultivation practices

Importance of the present work

- Due to the importance of Cassava Mosaic Disease and lack of resistance source, it is worth to develop a transgenic cassava through pathogen derived resistance.

- The present work emphasises on attempts to develop a strategy for engineering virus resistance in cassava plants through introduction of virus Replicase associated gene (AC1) in order to inhibit the transcription/translation

AC1 amplification strategy



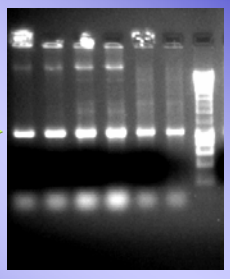
AC11 (N– terminal of AC1)

5'AAGCTTATGTCACCACCTAAGCGCTTTCAAATAAC 3'

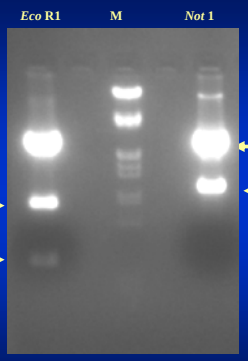
AC12 (C-terminal of AC1)

5'GGATCCTTAGCAGCTCTGTGTTGGACCTTGATTGG 3'

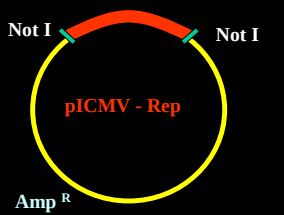
ICMV Replicase
(1.0kb)



Amplification of AC1 gene



Restriction of pICMV-Rep clone with *Eco* R I and *Not* I

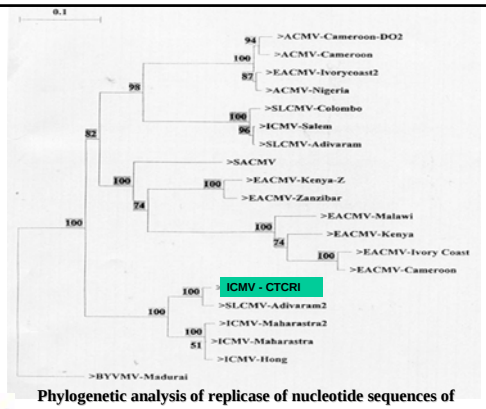


pGEM – T easy

GACCTTGATTGGGACCTGAGTATAATGGTTCGTCGAGGGAGATGAAGGTCGCA
TTTTTGATAGCCCAGGCTTTCAATGCGCTGTTTTTGTCTTCGTCCAAGAACTCTT
TATAGCTGGAATTGGGCCCTGGATTGCAGAGGAAGATAGTGGGAATACCTCCT
TTAATTTGAACTGGCTTTCCGTACTTGGTGTTTGATTGCCAGTCTCTCTGGGCCC
CCATGAATTCTTTGAAGTGCTTTAGGTAGTGGGGATCTACGTCATCAATGACGT
TGTACCACGCATTATTGTTGTATACCTTGGGACTTAAGTCCAGACGTCCACATA
GATAATTATGTGGACCCAATGCACGGGCCCACATAGTTTTACCTGTCCGACTAT
CGCCCTCGATTACGATTGACTTAGGTCTCAAGGGCCGCGCAGCGGCATCCATC
ACGTTTTCGTGAAACCACTCGTCGAGTTCCTCTGGAACTCGGTCAAATGAAGA
GAGGGGGAAAGGGTTCTGATACGGAGGTGGTGGTGTTGTAAAAATTCGATCCA
GGTTCGAGTTTATGTGATGGAAGTCTCTGAGGTAATCCCTAGGTGCTAATTCTT
TAAGGATCGTAAGAGCCGCTGATTTACTGCCGCTGTTAAGTGCTGCGGCGTAA
GCGTCGTTTGCAGATTGTTGACCTCCTCTTGCAGATCTTCCATCGATCTGGAAT
GTTCCCCATTCCAATGTATCGCCGTCTTTGTCGATGTAGGACTTGACGTCGGAG
CTGGATTTAGCTCCCTGAATGTTCGGATGGAAATGTGCTGACCTGGATGGAGA
TACCAGGTCGAAGAATCGCTGATTCTGGCATTTGTATTTTCCTTCGAACTGGAT
GAGCACGTGCAGATGAGGCTCCCCATTCTCATGGAGCTCTCTACAGATCTTGA
TGAATTTAGTGTTGGTGGGTGTTTGAAGCGCTCGAAGTTGTGAAAGTGCCTCTT
CTTTAGTGAGAGAGCACCTAGGATAAATGAGGAAATAGTTTTTGGCATTTATTT
GAAAGCGCTTAGGTGGTGACAT 1042

Nucleotide sequence ICMV AC1 gene

(GenBank Accession No. AY149901)

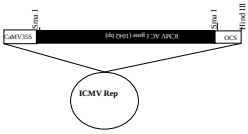


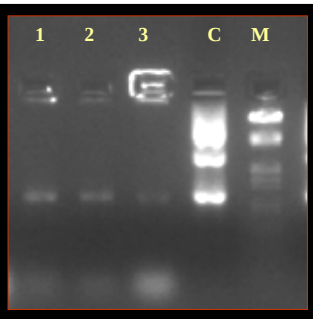
Phylogenetic analysis of replicase of nucleotide sequences of pICMV-Rep with other cassava mosaic viruses and begomoviruses

Recloning of AC1 gene in plant transformation vector

Gene insert - **Full length AC 1 (1042 bp) in sense and antisense direction**

Vectors - **pBin AR, pBI 121, pCAMBIA 2301**



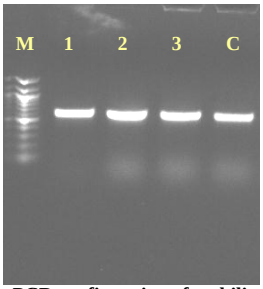


1,2, 3 –pBin 19/R in LBA 4404 with Rep

C- Control Rep in pGEM-T with Rep

M- molecular marker

Colony PCR confirmation of mobilisation of AC1 gene construct in LBA 4404 using AC1 gene specific primer



1,2, 3 –LBA 4404 having pCAMBIA 2301

C- Control (DNA of pCAMBIA 2301 vector)

M- molecular marker

Colony PCR confirmation of mobilisation of pCAMBIA 2301 in LBA 4404 using *npt*II primer (used for standardisation of transformation protocol)

PRODUCTION OF TARGET TISSUE FOR TRANSFORMATION

Micropropagation of H- 226 *In vitro* plants

CASSAVA TRANSFORMATION THROUGH *AGROBACTERIUM* MEDIATED GENE TRANSFER

Cassava cultivar: h-226

Strain used : LBA 4404

Constructs used:

- *Rep* in pBin AR
- pCambia 2301 (Vector alone for standardisation)

Media used : LB & YM

Explants: Cotyledons from 21 days old somatic embryos

Selection: Paromomycin (25mg/l)

STANDARDISATION OF PROTOCOL FOR AGROMEDIATED GENE TRANSFER

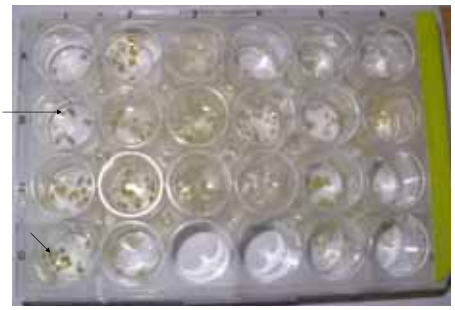
Protocol :

- 2 ml culture of *Agrobacterium* (24 hrs)
- 20 ml culture of *Agrobacterium* (12hrs) to reach OD value of 0.5 at 600nm
- Media used : YM /LB + Rif (50µl/l) +Kan(50 µl/l)
- Pelleting & incubation of cultures for 2 hrs (28°C) ,200 rpm in MS salt solution +Acetosyringone (100µM)
- Target Tissue used : Callus, Cotyledon explants,Variety : H 226
- Cocultivation for 2 hrs, removal of excess Agrobacterium and incubation for 48 hrs (28°C)
- Selection in MS medium supplemented with Picloram (50µM)+ Carbenicillin (500 mg/l) + Cefotoxime (200 mg/l) + Paromomycine (10-25 mg/l)
- Gus assay/ PCR using *Rep* and *npt II* gene primers

Standardisation of co-cultivation duration

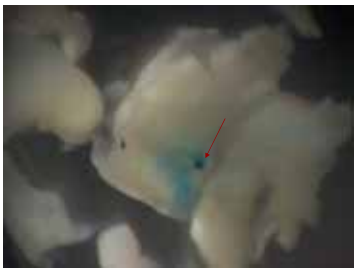
Co-cultivation duration	No. of explants used	No. of explants GUS positive
48 hr	50	16
72hr	50	7

Gus gene incorporation analysis



GUS assay of transformed cotyledons of cassava variety H-226 having pCAMBIA 2301 vector

GUS ASSAY

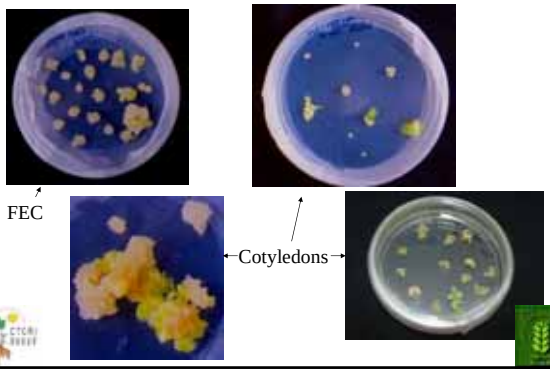


GUS assay of transformed cotyledons of cassava variety H-226 having pCAMBIA 2301 vector

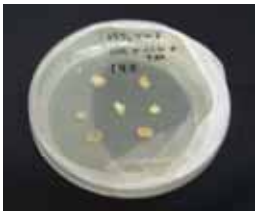
Transformation of cassava with ICMV *Rep* gene construct

- 750 explants used for transformation
- 93 explants survived in 10mg/l paromomycin selection
- 34 explants finally survived in 25mg/l paromomycin selection
- Transgenic tissues were multiplied & maintained in selection medium

Explants used for transformation



Multiplication of transformed calli



Initiation of somatic embryos from transformed calli

Paromomycin
(20mg/l)

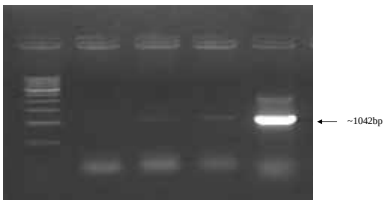
Paromomycin
(25mg/l)

Maturation of somatic embryo

Shoot development from matured somatic embryo

Shoot elongation

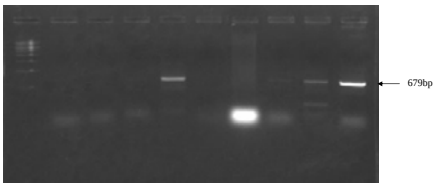
Gene incorporation analysis through PCR



Gene incorporation analysis using ICMV *Rep* primer in the transgenic cassava tissues incorporated with *Replicase* gene construct

- Positive result obtained for 6 lines

Gene incorporation analysis through PCR



Gene incorporation analysis using *npt II* primer in the transgenic cassava tissues incorporated with *Replicase* gene construct

Rooting

Hardening of transformed plants

Summary

- AC1 Gene was PCR amplified and cloned in pGEM-T vector
- AC1 gene was sequenced which had 1042 bp and which encoded for 347 amino acids (Gen Bank Acc.No. AY 149901)
- Recloning of *Rep* gene in pBinAR plant transformation vector in antisense direction.
- Protocol for transformation of cassava was standardised
- *Agrobacterium* mediated transformation were done with Rep gene constructs using cotyledon explants tissues
- Transgenic tissues were obtained and maintained in selection medium having 25mg/l paromomycin
- Gene incorporation analysis showed positive for *Rep* and *nptII* genes in PCR

Transgenic team at CTCRI….

Acknowledgement

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