ENGINEERING INDIAN CASSAVA FOR RESISTANCE AGAINST THE MOSAIC DISEASE

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CENTRE FOR PLANT MOLECULAR BIOLOGY
TAMIL NADU AGRICULTURAL UNIVERSITY
COIMBATORE - 641 003
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Thesis submitted in part fulfillment of the requirements for the award of the degree of DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY to the Tamil Nadu Agricultural University, Coimbatore

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(Anuradha T)
Engineering Indian cassava for resistance against the mosaic disease

By

Anuradha T

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2011

Abstract

Cassava is a major tuber crop grown in Tamil Nadu and Kerala. Cassava production is affected by a combination of biotic and abiotic stresses, among them; cassava mosaic disease (CMD) caused by the ICMV (Indian cassava mosaic virus) and SLCMV (Sri Lankan cassava mosaic virus) limit the productivity of cassava. None of the strategies now available is able to completely protect cassava against these viruses. Genetic engineering approach provides an attractive alternative strategy to engineer CMD resistance in cassava. As a pre-requisite to cassava transformation, a somatic embryogenesis based regeneration protocol was standardized in a popular but CMD-susceptible cassava variety, H-226. The immature leaf lobes were found to be the most suitable explant source for somatic embryo induction in vitro. The greatest frequency of embryogenic callus induction was recorded on MS medium containing B5 vitamins, 30 g/l maltose, 12 mg/l picloram and 4 g/l Phytagel. With a view to producing a large number of somatic embryos, secondary (cyclic) somatic embryogenesis was established using the mature green somatic cotyledonary stage embryos. The transfer of primary and secondary somatic embryos at cotyledon stage in MS regeneration medium containing 0.2 mg/l BAP and 0.01 mg/l NAA resulted in more than 80% embryo maturation. In order for elongation of the mature embryo, the three week-old, mature somatic embryos
were then transferred to a regeneration medium containing 0.2 mg/l BAP, 0.01 mg/l NAA and 0.2 mg/l GA₃. A total of 60 regenerated plants were hardened and established in greenhouse.

The infected cassava leaf samples from five major cassava growing districts of Tamil Nadu were analyzed for the presence of ICMV and SLCMV. SLCMV was predominant in samples from Salem districts, whereas ICMV was more prevalent in samples from Coimbatore district. In addition to individual virus infection, mixed infection of ICMV and SLCMV was observed in 23% of the samples tested.

Genetic engineering of cassava was attempted using RNAi technology. Four different RNAi vectors were constructed, utilizing a conserved 440bp of 5' end of ICMV and SLCMV 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 and SLCMV were cloned in sense and anti-sense orientations in the RNAi intermediate vector, pHANNIBAL. After cloning into pHANNIBAL, the cloned RNAi gene cassettes of ICMV and SLCMV were released and cloned into the binary vector, pART27, which contains the kanamycin-resistance gene as a plant selectable marker. These constructs were named pICR1 and pSCR1 for use against ICMV and SLCMV respectively. In order to use hygromycin as a selection agent in cassava genetic transformation, RNAi–Rep gene cassettes of ICMV and SLCMV were cloned into pCAMBIA1305.2. These constructs were named pICR2 and pSCR2 for use against ICMV and SLCMV respectively.

*Agrobacterium* mediated transformation of cassava was done with the RNAi constructs developed in the present study. Two different explants viz., immature leaf lobes and somatic cotyledons were used for co-cultivation. Transformed tissue selected on hygromycin (30 mg/l) for pSCR2 (SLCMV-specific) gene silencing construct. In total, 48 putative transgenic cassava shoots were regenerated on a regeneration medium containing 30 mg/l hygromycin, of which 2 putative transgenic plants were transferred to hardening. All the eight putative transgenic cassava plants were PCR-positive for hph gene and Rep gene indicating integration of transgenes of interest. *Agrobacterium*-mediated transformation of cassava with ICMV-specific gene silencing construct (pICR1) produced 15 regenerated shoots on kanamycin (100 mg/l); however, none of the regenerated shoots could survive up to hardening stage.
## CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER NO.</th>
<th>TITLE</th>
<th>PAGE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>REVIEW OF LITERATURE</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>MATERIALS AND METHODS</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>RESULTS</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>DISCUSSION</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>SUMMARY</td>
<td></td>
</tr>
</tbody>
</table>

REFERENCES

APPENDICES
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP</td>
<td>Benzylaminopurine</td>
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<td>bp</td>
<td>base pair</td>
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<tr>
<td>CaMV</td>
<td>Cauliflower mosaic virus</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>d</td>
<td>day(s)</td>
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<tr>
<td>2, 4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleoside triphosphate</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
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<tr>
<td>g</td>
<td>gram(s)</td>
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<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
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<td>h</td>
<td>hour(s)</td>
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<tr>
<td>kbp</td>
<td>kilo base pair</td>
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<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>l</td>
<td>litre</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<td>M</td>
<td>molar</td>
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<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>m.ha</td>
<td>million hectare</td>
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<tr>
<td>min</td>
<td>minute(s)</td>
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<td>ml</td>
<td>millilitre</td>
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<td>mm</td>
<td>millimetre</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>MS</td>
<td>Murashige and Skoog</td>
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<td>m.t</td>
<td>million tonnes</td>
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<td>Abbreviation</td>
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<tr>
<td>M.W.</td>
<td>Molecular weight</td>
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<td>NAA</td>
<td>naphthaleneacetic acid</td>
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<td>ng</td>
<td>nanogram</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
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<td>Ribonuclease A</td>
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<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA</td>
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<td>TE</td>
<td>Tris-EDTA</td>
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<td>wk</td>
<td>week(s)</td>
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<tr>
<td>YEP</td>
<td>Yeast extract peptone</td>
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<td>µg</td>
<td>microgram</td>
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<td>µl</td>
<td>microlitre</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>Table No.</td>
<td>Title</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Summary of Geminiviruses resistance strategies in crop plants</td>
</tr>
<tr>
<td>2.</td>
<td>Explants and culture methods used in cassava tissue culture</td>
</tr>
<tr>
<td>3.</td>
<td>Summary of genetic transformation in cassava</td>
</tr>
<tr>
<td>4.</td>
<td>Different media used for cassava somatic embryogenesis and regeneration</td>
</tr>
<tr>
<td>5.</td>
<td>District wise symptomatic cassava leaf sample collection from Tamil Nadu</td>
</tr>
<tr>
<td>6.</td>
<td>Primers designed for amplification of sense and antisense of Rep gene of CMV used for RNAi vector construction</td>
</tr>
<tr>
<td>7.</td>
<td>Influence of different concentrations of auxins on primary somatic embryo induction from immature leaf lobes of cassava variety, H226</td>
</tr>
<tr>
<td>8.</td>
<td>Effects of different media composition on embryogenic calli induction from immature leaf lobes of cassava variety, H226</td>
</tr>
<tr>
<td>9.</td>
<td>Secondary somatic embryogenic in cassava somatic cotyledons with different concentrations of Picloram</td>
</tr>
<tr>
<td>10.</td>
<td>Effect of different concentrations of BAP and NAA on somatic embryo maturation in cassava</td>
</tr>
<tr>
<td>11.</td>
<td>Effect of different concentrations of GA$_3$ on somatic embryo regeneration in cassava</td>
</tr>
<tr>
<td>12.</td>
<td>Accession numbers of Rep gene of ICMV and SLCMV from Tamil Nadu deposited in NCBI</td>
</tr>
<tr>
<td>13.</td>
<td>Identification of CMD causing virus through PCR screening of symptomatic cassava leaf sample from Tamil Nadu</td>
</tr>
<tr>
<td>14.</td>
<td><em>Agrobacterium</em> mediated genetic transformation of somatic cotyledons of cassava with RNAi construct</td>
</tr>
<tr>
<td>15.</td>
<td><em>Agrobacterium</em> mediated genetic transformation of immature leaf lobes of cassava with RNAi construct</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Genome model of DNA A and DNA B of a bipartite Begomovirus (ICMV/SLCMV)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Field distribution of CMD in Tamil Nadu</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Phylogenic tree of cloned ICMV Rep gene isolates from Tamil Nadu</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Phylogenic tree of cloned SLCMV Rep gene isolates from Tamil Nadu</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Physical map of pCR1 and pSCRI</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Physical map of pCR2 and pSCR2</td>
<td></td>
</tr>
</tbody>
</table>
## List of Plates

<table>
<thead>
<tr>
<th>Plate No.</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Primary somatic embryogenesis from immature leaf lobes of cassava variety, H226</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Regeneration of cassava from immature leaf lobes of cassava variety, H226</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Cyclic somatic embryogenic induction and regeneration from somatic cotyledons of cassava variety, H226</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Cloning of ICMV and SLCMV Rep gene from symptomatic leaves of cassava</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Construction of ICMV Rep-RNAi construct in pART27 binary vector</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Confirmation of pICR1 vector in <em>Agrobacterium</em> strain LBA4404</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Construction of Rep-RNAi construct of ICMV in pCAMBIA 1305.2 binary vector</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td><em>Agrobacterium</em> mediated transformation of cassava somatic cotyledons with SLCMV-Rep-RNAi construct</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td><em>Agrobacterium</em> mediated transformation of cassava immature leaf lobes with ICMV-Rep-RNAi construct</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>PCR analysis of putative transgenic lines of Cassava transformed with SLCMV specific RNAi cassette</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>PCR analysis of putative transgenic lines of Cassava transformed with ICMV specific RNAi cassette</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

Cassava (*Manihot esculenta*) is a major tuber crop cultivated in 13 states of India. Cassava is mainly grown in large area in the Salem and Dharmapuri District of Tamil Nadu and grown throughout Kerala. 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) (FAO STAT, 2010).

Cassava production is affected by a combination of biotic and abiotic stresses, among them; cassava mosaic disease (CMD) caused by the Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV) 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 virus 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. There are six species of Geminivirus that cause CMD in Africa (Fauquet and Stanley, 2003). Mixed infection of ICMV and SLCMV is often involved in causing CMD in India (Patil *et al.*, 2005). It is reported that Geminivirus species simultaneously infecting the same plant can lead to very severe yield reductions and even crop failure (Pita *et al.*, 2001a; Calvert and Thresh, 2002).

Cassava regeneration and transformation protocol has been established well in few African varieties. Regeneration of plants from germinating somatic embryos induced on cotyledons of zygotic embryos, immature leaves or primary somatic embryos has been the only reproducible method of *de novo* regeneration of cassava *in vitro* (Stamp and Henshaw 1982, 1987; Szabados *et al.*, 1987; Mathews *et al.*, 1993; Raemakers 1993, 1997, 2001). Regeneration from embryogenic suspensions has also been
Transformation and regeneration of transgenic cassava plants expressing kanamycin resistant gene as selectable marker has been reported by several laboratories (Sarria et al., 1993, 1995, 1997; Li et al., 1996; Raemakers et al., 1996; Schopke et al., 1996; Gonzalez et al., 1998; Taylor et al., 2004; Zhang et al., 2005; Vanderschuren et al., 2007; Bull et al., 2010). However, there is no report so far available for the regeneration as well as stable genetic transformation for Indian cassava varieties.

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 for viral disease resistance, with both groups employing pathogen-derived resistance (PDR) strategies. At ETH the approaches for the control of geminivirus include the expression of antisense viral sequences which interrupt the virus proliferation at the level of DNA replication (Zhang et al., 2003; Vanderschuren et al., 2009). Many reports have demonstrated that RNAi can be engineered to target viral RNA in plants (Smith et al., 2000; Tenllado et al., 2000). 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., 2003. Furthermore, a PTGS based strategy to control DNA virus replication was demonstrated when plant cells simultaneously transfected with African Cassava Mosaic Virus (ACMV) and with a synthetic siRNA designed to target the AC1 gene of the virus showed a reduction in the accumulation levels of AC1 mRNA by more than 90% and viral DNA by 70% compared with controls (Vanitharani et al., 2003). The DNA-A of the CMV codes for the AC1 gene, the replication-associated protein gene (or \textit{Rep} gene), which is indispensable for the replication of virus and disease development. The transgenic cassava expressing the full length AC1 gene from African Cassava Mosaic Virus (ACMV) imparts resistance against the virus (Chellappan et al., 2004a) demonstrating that RNAi targeting the \textit{Rep} gene is a promising method for viral resistance in cassava.

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 \textit{Rep} gene, which is indispensable for DNA replication of virus (Pooggin et al., 2003).

With this background, the present study aims at developing transgenic cassava resistant to Cassava mosaic virus through RNAi approach. As cassava is a recalcitrant crop to genetic transformation, well standardized protocol towards transformation and regeneration is required. The first part of the study
was to initiate somatic embryogenesis in elite genotype of Indian cassava (H226) and subsequent multiplication of the embryogenic tissue by secondary/cyclic somatic embryogenesis, followed by regeneration. The second aspects of the study aims at cloning and characterization of Indian and Sri Lankan cassava mosaic virus in Tamilnadu followed by constructing gene silencing vector for CMV control in cassava. The final part deals with the transformation and recovery of transgenic cassava plants.

The present study was carried out with following objectives

1. Standardization of regeneration protocol in cassava variety, H-226
2. Isolation and characterization of Rep gene of ICMV and SLCMV from Tamil Nadu.
3. Construction of RNAi-Rep gene silencing vectors for control of ICMV and SLCMV
4. Genetic transformation of cassava variety, H-226 for CMD resistance
CHAPTER II
REVIEW OF LITERATURE

2.1. History of the disease and its distribution

Cassava mosaic disease (CMD) was first reported in 1894 by Warburg, in Tanzania. Zimmerman (1906) gave the first suggestion that it might be caused by a viral pathogen. In India it was first recorded in 1956 by Abraham and the disease was described later by Alagianagalingam & Ramakrishnan (1966). It is reported that there are three distinct groups of cassava mosaic virus, grouped as Group A, B and C through monoclonal antibody screening (Harrison et al., 1991). They found that group C isolates were reacting only with two to three MAbs (Monoclonal Antibodies) and they named the virus as Indian Cassava Mosaic Virus. Further these three distinct cassava mosaic virus groups were renamed as African cassava mosaic virus-K (Group A), East African cassava mosaic virus (EACMV) and Indian cassava mosaic virus (ICMV) respectively by nucleotide sequence comparisons (Hong et al., 1993). Later a new cassava mosaic virus was reported in Sri Lanka and it was named as Sri Lankan Cassava Mosaic Virus (Saunders et al., 2002). So far there are eight distinct cassava mosaic virus species approved by Geminiviridae study group.

2.2. Symptomatology:

Infected plant showed stunted growth and the leaves showed mosaic mottling. In fully developed diseased leaves, the chlorotic areas were yellow intermingled with normal green colour (Alagianagalingam & Ramakrishnan, 1966). The disease significantly reduced the stem girth, plant height, leaf length and width in different cassava varieties (Thankappan and Chacko, 1976). The leaf area was reduced in extreme cases; leaf distortion and shoestring appearance were also observed (Jos et al., 1984; Mathew, 1989).

2.3. Disease incidence and yield loss:

The incidence of CMD was generally high in coastal and western Kenya, where it exceeded 80% in some districts and approached 100% in some individual plantings (Bock, 1983). The Infection was also high in a sample of 10 farms assessed in Ghana, where the mean incidence was 96% (Walker et al., 1985). In India it is reported that the overall incidence of CMD was 23% in Kerala and 30% in Tamil Nadu, which was very high compared with Andhra Pradesh (less than one per cent) and Karnataka (5%) (Mathew, 1989).
It is reported that restriction of virus movement into axillary buds occurred in the all-resistant and moderately resistant genotypes (Ogbe et al., 2002). The virus concentration in the CMD resistant cassava genotypes and susceptible genotypes was similar where the resistant genotypes had significantly lower symptom severity scores than the susceptible genotypes and the resistant and moderately resistant genotypes that had high virus concentrations sustained storage root yield losses (Ogbe, 2003). Yield loss up to 86% has been recorded in the susceptible cultivar F279 in Kenya (Bock and Guthrie, 1978).

Thresh et al. (1997) estimated losses in Africa due to CMD is of between 15% and 24%, which is equivalent to 15-27 Mt per annum. It is observed that the reduction in tuberous root weight was greatest in plants dually infected with ACMV and EACMV-UG2, averaging 82%. Losses attributed to ACMV alone, EACMV-UG2 mild strain and EACMV-UG2 severe strain were 42%, 12% and 68%, respectively (Owor et al., 2004). The overall yield lose due to ICMV infection was reported to be of 18 – 25% in India (Dasgupta et al., 2003).

2.4. Transmission of the virus:

2.4.1. Transmission of cassava mosaic viruses by the whitefly *Bemisia tabaci*

The first whitefly transmission of cassava mosaic virus was reported in Congo by Ghesquière in 1932 and the whitefly species referred as *Bemisia mosaicivecta* which was later confirmed that it was the misprint of *B. mosaicivectura* (Storey & Nichols, 1938). In India, Alagianagalingam & Ramakrishnan in 1966 reported *B. tabaci* as vector of ICMV. The whitefly transmission rate was found to be of 19 % from cassava to cassava, and from cassava to *Nicotiana tabacum* cv. Jayasri (100%), *N. rosulata* (67%) and 11 other *Nicotiana* spp. (20-50%) using 50 whiteflies per test plant (Mathew and Muniyappa, 1993). It is found that the CP gene is intimately involved in insect transmission of ACMV by changing insect specificity by altering coat protein gene with beet curly top virus which is transmitted by leaf hopper through chimeric mutants combining the ACMV genome with the coat of the beet curly top virus resulted mutants transmitted by leaf hopper (Briddon et al., 1990).

2.4.2. Mechanical transmission:

Malathi and Sreenivasan (1983) and Malthi and Nair (1984) reported transmission of ICMV from cassava to *N.benthamiana* and *N. glutinosa* by using partially purified preparations but transmission by crude sap was not successful. The virus was not transmitted from cassava to cassava or from *N.benthamiana* to cassava.
But the sap transmission of ICMV from cassava to *Datura stramonium*, *Nicotinia* spp. and *Petunia hybrida* was 80% and from *N. benthamiana* to *N. benthamiana* was 100% (Mathew and Muniyappa, 1993). ICMV was transmitted more efficiently by sap to *N. benthamiana* rather than through whiteflies to cassava.

### 2.5. Genome organization of Cassava Mosaic Virus

The majority of whitefly transmitted geminiviruses have bipartite genomes with A and B components. The two components share only a `common region ' (CR) of approximately 200 bp with high sequence identity (90±100%). The CR contains promoter and sequence elements required for DNA replication and transcription (Chatterji *et al.*, 1999; Eagle *et al.*, 1994; Laufs *et al.*, 1995; Zhan *et al.*, 1991). Component A encodes all viral proteins necessary for replication and encapsidation of both DNAs (Rogers *et al.*, 1986; Stanley, 1983; Sunter *et al.*, 1987; Townsend *et al.*, 1986; Maheshkumar *et al.*, 2005). The DNA-A components of *Cassava mosaic virus* consist of six genes: AC1 codes for a replication-associated protein (Rep), AC2 codes for a transcriptional activator protein(TrAP), AC3 codes for a replication enhancer protein (REn), AV1 codes for the coat protein (CP), AV2 codes for the precoat, and AC4 codes for a protein to which no function has yet been attributed (Hanley Bawdon *et al.*, 1999; Padidam *et al.*, 1996; Sunter and Bisaro, 1992; Dutt *et al.*, 2005). However, in monopartite geminivirus C4 a positional homologue of AC4 is a major determinant of pathogenesis (Vanitharani *et al.*, 2004) (Figure.1).

Rep initiates viral DNA replication by binding specifically to reiterate motifs (iterons) within the intergenic region (Fontes *et al.*, 1994) and introducing a nick into the conserved TAATATT/AC sequence (Heyraud-Nitschke *et al.*, 1995). Rep also binds to the plant homologue of retinoblastoma protein (Rb) to regulate cell-cycle progression; altering the environment of terminally differentiated cells to provide host factors that support viral DNA replication (Kong *et al.*, 2000). TrAP transactivates expression of virion-sense gene expression from both DNA-A and DNA-B (Sunter and Bisaro, 1992) and also functions in the suppression of post-transcriptional gene silencing (Voinnet *et al.*, 1999). The REn protein, although not essential, will boost viral DNA replication several fold (Sunter *et al.*, 1990). The NSP and MP proteins coded by the DNA-B component are essential to shuttle viral proteins and DNAs from the cytoplasm to the nuclei and from one cell to the next (Sanderfoot and Lazarowitz, 1995).
Figure 1. Genome model of DNA-A and DNA-B of a bipartite virus (ICMV/SLCMV)
2.6. Variability studies and evolution of new viruses and its strains through recombination

In 1997, Deng et al and Zhou et al reported individually that severe form of CMD was caused by a recombinant of ACMV and EACMV in which approx. 400 nucleotides of the coat protein (CP) gene of ACMV replace the similar region of EACMV after the reports of the rapid spread of an unusually severe form of CMD in central Uganda (Gibson et al., 1996 and Otim-Nape et al., 1996). And then the recombinant was designated EACMV-UG2 on the basis of its more than 90% homology in the DNA-A sequence with EACMV (Deng et al., 1997; Pita et al., 2001a; Fauquet & Stanley, 2003) which was reported to be responsible for the severe epidemic of CMD (Harrison et al., 1997; Otim-Nape et al., 1997; Legg, 1999). Recently it had definitive designation as EACMV-UG confirmed after a comprehensive review of the taxonomy of the family Geminiviridae (Fauquet and Stanley, 2003).

Recombination is a significant contributor to geminivirus evolution. The high rate of recombination may be contributing to the recent emergence of new geminivirus diseases and it is revealed that recombination is very frequent and occurs between species and within and across the genera of the geminiviruses (Padidam et al., 1999)

Nicotiana benthamiana was transformed with three different constructs containing the coat protein coding sequence of African cassava mosaic virus (ACMV). Transformed plants were inoculated with a coat protein deletion mutant of ACMV that induces mild systemic symptoms in control plants. Several inoculated plants of transgenic lines developed severe systemic symptoms typical of ACMV. DNA analysis revealed that, in these plants, recombination had occurred between the mutant viral DNA and the integrated construct DNA, resulting in the production of recombinant virus progeny with ‘wild-type’ characteristics (Frischmuth and Stanley, 1998)

In 2000 Fondong et al reported synergistic interaction between the Cameroon isolates of ACMV (ACMV/CM) and EACMV (EACMV/CM) as they observed Nicotiana benthamiana plants were doubly inoculated with these two viruses by using sap from cassava plants or infectious clones, shown the more severe symptoms than for plants inoculated with either virus alone.

It is reported that recombination, pseudo recombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda as they found new strain of EACMV – UG3 DNA B associated with EACMV – UG2 DNA A which evolved due to recombination between ACMV and EACMV – UG1 (Pita et al., 2001). They further reported that a synergistic interaction
between ACMV–UG and EACMV-UG1. Berrie et al (2001) reported that SACMV is most closely related to EACMV in both of the DNA A and B.

The Srilankan cassava mosaic virus (SLCMV) DNA A has biological characteristics of a monopartite begomovirus, and the virus was probably evolved by acquisition of a DNA B component from ICMV, as sequence comparisons suggest that SLCMV DNA B originated from ICMV DNA B by a recombination event involving the SLCMV DNA A intergenic region (Saunders et al., 2002).

AC4 of Cameroon strain of African cassava mosaic virus (ACMV- [CM]) has synergetic effect on disease severity when combined with AC2 region of EACMV due to its suppression of host’s post transcriptional gene silencing against these viruses and the same thing happened between AC4 of Sri Lankan cassava mosaic virus and AC2 of Indian cassava mosaic virus as suppressors of PTGS (Vanitharani et al., 2004).

A new species of CMV with mild phenotype was reported and named as East African cassava mosaic Zanzibar virus (EACMZV) as the virus has maximum nucleotide identity of 83% in the DNA-A component with other sequenced begomoviruses, together with different biological properties, once again it was claimed that this new virus was evolved by recombination (Maruthi et al., 2004). Both ICMV and SLCMV were present in mosaic-affected cassava in India and they exhibited a high variability of up to 40% was detected by PCR and RFLP analysis (Patil et al., 2005). Recently it is reported that most of the sequenced representative examples (7 of 8) are more related to SLCMV and only one representative example has the similarity with ICMV and all reported isolates are all variants of SLCMV and ICMV from Southern India (Rothenstein et al., 2005). Further, it is confirmed that SLCMV is present in India (Kerala) and it is the only second virus causing cassava mosaic next to ICMV in India (Dutt et al., 2005).

It is reported that EACMV-UG2 was the predominant virus and occurred in all the surveyed regions of Uganda. It was detected in 73% of the severely and 53% of the mildly diseased plants, while ACMV was less widespread and occurred most frequently in the mildly diseased plants. Mixed infections of ACMV and EACMV-UG2 were detected in only 18% of the field samples (Sseruwagi et al., 2004). East Africa is considered as a source of diversity and evolutionary change for CMGs and in more recent times based on molecular analysis, Tanzania is considered as center of diversity in particular (Ndunguru et al., 2005).
Presently eight cassava mosaic viruses (six from Africa and two from Indian subcontinent) have been approved by Geminiviridae Study Group based on the sequence homology demarcation between species as 89% for the DNA-A component of begomoviruses (Fauquet and Stanley, 2003).

2.7. Molecular characterization of Cassava mosaic virus

2.7.1. Detection and differentiation of SLCMV and ICMV infection through Multiplex PCR

To distinguish between SLCMV and ICMV, Patil et al. (2005) designed the primers to specifically amplify a 904 bp fragment from ICMV and a 599 bp fragment from SLCMV encompassing the 5’ portion of AC1 and the intergenic region. Thus, amplification reactions containing both the discriminatory and the common primers amplified a 904 bp fragment if only ICMV was present, a 599 bp fragment if only SLCMV was present and both the above fragments, if both the viruses were present.

Manivasagam (2006) reported that out of the fifty Cassava mosaic virus infected samples analyzed only two samples one from Musiri (Trichy district) and other from Mallur (Salem district) were detected with ICMV infection as 904 bp fragment of DNA-A was amplified. All the other samples from various districts of Tamil Nadu were detected invariably with SLCMV as they amplified 599 bp of DNA A. Dual infection of both ICMV and SLCMV was not observed in any of the sample.

Berry and Rey (2001) developed a technique to test viral-infected cassava called as heteroduplex mobility assay (HMA) which was involved in the amplification of highly conserved core region of the coat protein gene of field isolates of cassava infecting begomoviruses followed by denaturing and annealing with a number of reference strains. The HMA profiles in this study were able to differentiate four different viral species including ACMV, EACMV and SACMV and 11 different virus strains, and showed a good correlation with sequencing results and phylogenetic comparisons with other sequenced cassava viruses.

2.7.2. Amplification of DNA-A and DNA-B of Cassava mosaic virus

Full length DNA-A and DNA-B components of ICMV were amplified by polymerase Chain Reaction. Amplified products were obtained from all the infected samples, but not from the non infected sample (Rothenstein et al., 2005).

DNA-A and DNA-B genomic components were amplified from the cassava mosaic virus infected samples by using the primers specifically designed for ICMV (Dutt et al., 2005). Saunders et al.,
2002 amplified 2.7 kb of ICMV and SLCMV DNA-A, DNA-B from the Cassava mosaic virus infected samples. The full-length DNA-A and DNA-B of ACMV was amplified using the primers JSP004, JSP005, JSP008, JSP009 while primers, VNF003, VNF004, EB03, EB04 were used to amplify full-length molecules of EACMV-CM/IC DNA-A and –B (Pita et al., 2001a).

DNA-A of cassava-begomoviruses viz., East African cassava mosaic virus (EACMV), East African cassava mosaic virus – Uganda variant (EACMV-UG), African cassava mosaic virus(ACMV), and East African cassava mosaic Zanzibar virus (EACMZV) was amplified form samples collected from Kenya, Uganda, and Democratic Republic of the Congo (DRC) (Were et al., 2004a).

Berrie et al. (2001) amplified 2.8 kb size of DNA-A and DNA-B of SACMV through PCR. Zhou et al. (1997) amplified whole DNA-A of Tanzanian isolate of EACMV. Full-length DNA-A (2785 nucleotides) and DNA-B (2763 nucleotides) components of EACMZV were subsequently PCR-amplified (Maruthi et al., 2004). A number of ICMV and SLCMV DNA-A components have been sequenced (Hong et al., 1993; Saunders et al., 2002; Dutt et al., 2005; Rothenstein et al., 2005; Rothenstein et al., 2006) and variation among these components has been shown to be relatively very low.

2.7.3. Amplification and cloning of AC1, AC2, AC3 and AC4 genes of Cassava mosaic virus

The genes AC2 and AC4 from SLCMV and ICMV, AC1,AC2, AC3, and AC4 of ACMV, AC1, AC2, AC3 and AC4 of EACMCV were PCR amplified with Platinum pfx DNA polymerase (Invitrogen) by using specific primers with restriction enzyme sites (Vanitharani et al., 2004). Makeshkumar et al. (2005) amplified 1042 bp size of AC1 gene from ICMV infected cassava samples. Patil et.al. (2005) amplified AC3 gene from ICMV and SLCMV infected cassava samples. Ogbe et al. (2003c) amplified AC2 and AC4 genes of EACMV. Zhou et al. (1998) amplified AC1 gene of EACMV. Manivasagam (2006) cloned and sequenced the partial DNA-A of Cassava Mosaic Virus infected samples collected from Salem and he reported that the cloned fragment shared maximum sequence identity with ICMV at nucleotide levels (93%) than with SLCMV (88 %)

2.8. Genetic engineering for virus resistance

Plant virus diseases cause severe constraints to the production of wide range of economically important crops throughout the world and consequently plant pathologists have devoted considerable efforts towards controlling viral diseases during twentieth century. Plant virus diseases were difficult to
control and the control measures normally taken by the use of insecticides to kill insect vectors, the use of virus-free propagating materials and the selection of plants with appropriate resistance genes. Virus free stocks were obtained by virus elimination through heat therapy/meristem tissue culture, but this approach was ineffective for viral diseases transmitted by vectors, while insecticides can control vectors but often the virus has already been transmitted to the plants before the insect vector is killed. The use of resistant cultivars is the most effective means of control; however their introgression into same crop is not possible.

Rapid advances in the techniques of molecular biology have resulted in the cloning and sequence analysis of the genomic components of a number of plant viruses. A majority of plant viruses have a single-stranded positive sense RNA as the genome. However, some of the most important viruses in tropical countries like India have single-stranded and double-stranded DNA genomes and RNA genomes of ambisence polarity, i.e. genes oriented in both directions. Concomitantly, tremendous advances have taken place in our understanding of plant–virus interaction in the process of pathogenesis and resistance. This, along with associated advances in the genetic transformation of a number of crop plants, has opened up the possibility of an entirely new approach of genetic engineering towards controlling plant virus diseases.

There are mainly two approaches for developing genetically engineered resistance depending on the source of the genes used. The genes can be either from the pathogenic virus itself or from any other source. The former approach was based on the concept of pathogen-derived resistance (PDR).

### 2.9. Pathogen-derived resistance (PDR)

The prospects for pathogen-mediated intervention in virus disease development were first realized in 1929 when Mckinney demonstrated that tobacco could be protected from infection by a strain of TMV by prior inoculation with a milder strain of TMV (Gadani et al., 1990). This type of protective measures known as cross-protection has since been employed throughout the world on several important crops, including tomato, papaya and citrus (Beachy et al., 1990; Gadani et al., 1990; Hull and Davies, 1992).

A problem of particular concern is that strain of viruses show variation in their virulence on different crops and even within varieties of the same crop. A virus used to protect one crop could potentially causes serious disease on other crops or varieties nearby. Several theories have been proposed to protect plants from virus infection.
Hamilton (1980) first postulated the concept of pathogen-derived resistance in plants and this concept were substantiated by Sanford and Johnston (1985) where they suggested that the transgenic expression of pathogen sequences might interfere with the pathogen itself. Sanford and Johnston (1985) developed the simple concept of parasite or pathogen-derived protection using transgenic plants. The PDR proposes that the expression of certain genes of a pathogen, in this case a virus, in a host would disrupt the normal balance of viral components and thereby interfere with the virus life cycle. Hence, such type of disruption might prevent the replication and/or movement of the virus beyond the initially infected cell. Even with less effective interference in the replication cycle, PDR might modulate the disease symptoms and result in only a localized infection. The first demonstration of PDR against plant viruses was given by Powell et al. (1986) who has shown that the expression of TMV coat protein in tobacco plants has protected those plants against TMV. Currently, there are two basic molecular mechanisms by which PDR is thought to operate, protein-based protection (protein-based-mediated resistance) in which the expression of an unmodified or modified viral gene product interferes with the viral infection cycle and nucleic acid-based protection, which does not involve the expression of a protein product (RNA-based-mediated resistance).

The realization that gene silencing is involved in PDR has evolved through several stages. There was no correlation between level of RNA accumulation and degree of resistance (Lawson et al., 1990). Subsequently resistance was conferred by modified viral transgenes that encoded untranslatable RNAs (de Haan et al., 1992; Dougherty, 1992c). This post-transcriptional mechanism operates at the RNA level and would therefore have the potential to suppress the accumulation of viral RNA that shares sequence identity with the silenced transgene.

2.9.1. Protein-based-mediated resistance

2.9.1.1 Coat protein (CP)-mediated protection

The use of viral CP as a transgene for producing virus resistant plants was one of the most spectacular successes achieved in plant biotechnology. The coat protein gene of TMV was used first in the demonstration of virus-derived resistance in transgenic plants (Powell et al., 1986). They suggested that the transgenic tobacco plants expressing high level of TMV CP were more resistant to TMV virions that to TMV RNA. It was suggested that CP-mediated protection against TMV was through the inhibition of virion disassembly in the initially infected cells. It was proposed that RNA inoculum could overcome the
resistance because disassembly was not required to establish infection by naked RNA. The most important success story related to coat protein mediated resistance to a virus is against PRSV (*Papaya Ring Spot Virus*). Transgenic papaya (var. sunset) with CP gene was grown from 1991 to 1993, and remained virus-free for 25 months. Subsequently, it was further crossed with other popular varieties. One such variety, called Rainbow, yielded 112,000 kg/ha marketable fruits in 1995, compared to 5,600 kg/ha from non-transgenic lines (Chen et al., 2000).

2.9.1.2. Movement protein-mediated protection (MPMP)

Movement proteins (MP) were essential for cell-to-cell movement of plant viruses. These proteins have been shown to modify the gating function of plasmodesmata, thereby allowing the virus particles or their nucleoprotein derivatives to spread to adjacent cells. Coat protein-mediated protection is through the expression of wild type CP gene whereas MPMP is based on the transgenic expression of dominant negative mutant forms of viral genes. Transgenic expression of dysfunctional MP conferred resistance to TMV MP (Lapidot et al., 1993; Malyshenko et al., 1993). Resistance conferred by transgenic expression of a dysfunctional TMV MP is likely due to competition for plasmodesmatal binding sites between the mutant MP and the wild-type MP of the inoculated virus (Lapidot et al., 1993). MPMP exhibited broad-spectrum resistance. The protection conferred by the mutant MP of TMV mediates resistance to viruses of the potex-, cucumo-, and tobraviral groups in addition to the targeted tobamoviruses (Cooper et al., 1995). This suggested that MPs of different viruses might interact with the plasmodesmatal components (Carrington et al., 1996).

2.9.1.3. Replicase-mediated resistance (RNA dependent RNA polymerase resistance)

Gene constructs of Rep genes that have been used for resistance include full-length, truncated or mutated genes. This type of resistance remains confined only to a narrow spectrum of viruses. However, the resistance generated by the use of Rep sequences is very tight; a high dosage of input virus can be resisted easily by the transgenic plant.

Replicase protein-mediated resistance against a virus in the transgenic plant was first shown against TMV in tobacco plants containing a putative Rep gene encoding 54 kDa replicase protein (Golemboski et al., 1990). Similar resistance had developed for several viruses viz., pea early browning virus (MacFarlane and Davis, 1992), PVY (Audy et al., 1994) and CMV (Hellwald and Palukaitis, 1995). In plants carrying a transgene derived from the replicase genes of PVX (Muller et al., 1995), cowpea mosaic virus (Sijen et al.,
and pepper mild mottle tobamovirus (Tenllado et al., 1996), it is clear that there was a RNA-mediated, homology dependent resistance.

### 2.9.1.4. Antisense RNA in homology-dependent resistance

Development of conceptual models of PTGS and PDR to viruses, proposed mechanisms for suppressing the accumulation of nucleus-derived RNA (gene silencing) and virus-derived RNAs with homology to the transgene. Such a mechanism would require a high degree of sequence specificity, because resistance is highly strain specific.

In principle, the interaction leading to suppression of viral RNA could involve base pairing of the sense RNA transcript of the transgene and the negative strand of the viral RNA, which is produced as an intermediate in the replication cycle of most viral RNAs. The antisense RNA could be produced by an RdRp encoded in the host genome using the transgene RNA as a template (Schiebel et al., 1993a; 1993b) and would have the potential to base pair with the transgenic and viral RNAs.

Formation of duplex RNA could influence accumulation of host and viral RNA to cause gene silencing and virus resistance. The base paired region may render the duplex RNA susceptible to degradation by RNases specific for double-stranded RNA (Nicholson, 1996). The base-paired region could also arrest the translation and consequently have an indirect effect on RNase susceptibility and translation could cause reduced accumulation of both nucleus- and virus-derived RNAs. The proposed involvement of antisense RNA can be considered as a part of the response of homology-dependent resistance.

Reports of replicase-mediated resistance to PVY (Audy et al., 1994) and AMV (Bredearde et al., 1995) emphasised that the resistance mechanism was protein-based rather than RNA-based because the resistance phenotype was influenced by mutations affecting the primary structure of the protein encoded by the transgene. Transgene encoding wild-type version of the replicase protein did not confer resistance, whereas those encoding mutant proteins conferred strong resistance. Replicase-mediated resistance remains confined to a narrow spectrum of viruses, the spectrum being narrower than that of CP-mediated resistance. Recently transgenic cassava plants with increased ACMV resistance was developed through antisense RNA technology (Zhang et al., 2004.) In yet another study the full length replicase gene was modified by a mutation at its N-terminal and integrated into the cassava genome. This mutated rep gene
was found to impart high resistance to ACMV while compared to the wild rep gene which was shown to provide only moderate levels of resistance (Vanitharani, 2004).

2.9.1.5. RNAi (RNA interference)-mediated resistance

Sequence specific RNA degradation is described as PTGS (Post Transcriptional Gene Silencing) in plants, quelling in fungi and RNA interference in animals. Dougherty (1992) who demonstrated that untranslatable viral RNA sequence could trigger specific, post-transcriptional RNA degradation of the mRNA and are correlated with viral protection. Operationally, PTGS is observed as high-level nuclear transcription determined from nuclear run-on experiments. There was a low and steady state levels of mRNA accumulation in the cytoplasm as detected by northern hybridization. Therefore, the gene silencing system is triggered in the cytoplasm together with any viral RNA that has same or similar sequence. Hence, it is now revealed that many examples of resistance previously described to expression of viral genes such as coat-protein, replicase, antisense, sense co-suppression, satellite RNA, ribosome etc., are functionally operated by PTGS.

RNA silencing is an evolutionary conserved mechanism protecting cell from pathogenic RNA and DNA, which is increasingly viewed as an adaptive immune system of plants against viruses (Voinnet, 2001). Proof of the concept that RNAi can be engineered to effectively target geminiviruses has been documented in transient assays, initially for Mungbean yellow mosaic virus-Vigna (MYVVig; Pooggin et al., 2003) and later for African cassava mosaic virus (ACMV; Vanitharani et al., 2003). Transgenic tobacco plants that constitutively express double-stranded dsRNA cognate to coding and non-coding regions of DNA were developed against Cotton leafcurl virus (CLCuV) (Mansoor, 2003). These plants were resistant to CLCuV when challenged either by agroinoculation or by white fly transmission.

Transgenic *N. benthamiana* plants carrying two different 35S promoter-driven RNAi constructs were designed to target Tomato chlorotic mottle virus (ToCMV-[BR]) was produced in 2003. In this, one construct contained inverted repeat (IR) of the bi-directional DNA A promoter flanked with the 5′-parts of Rep (AC1) and CP (AV1) coding regions, while the other carried IR covering the entire Rep, TrAP (AC2) and Ren (AC3) coding regions. These transgenic plants (R1 generation) when agro inoculated with ToCMV showed significant delays in symptom development. However, none of the several transgenic lines tested was completely resistant to ToCMV. Also there was no correlation between the timing of delayed symptom appearance and the levels of small interfering RNA (siRNAs) accumulation (Ribeiro and Prins, 2003).
Rezaian et al., (2003) developed transgenic RNAi tomato plants with a *Tomato leaf curl virus* (TLCV) C2 intron-spliced hairpin construct that accumulated high levels of siRNAs cognate to the C2 ORF. Inoculation of these plants with TLCV resulted in delayed accumulation of viral DNA, correlating with high level of methylation of a complementary-sense strand of the dsDNA form. However, complete resistance (immunity) to the virus was not achieved. They could obtain the evidence that siRNAs corresponding to both coding and non-coding regions of TLCV and Tomato leaf curl Sardinia virus (TYLCSV) accumulate during normal virus infection in tomato and tobacco plants. Similar observations were also made in a model of ACMV-*N. benthamiana* system (Dodar et al., 2006). It has been also found for MYMV*Vig* (Trinks et al., 2004) and in Tomato golden mosaic virus (Bisaro, 2005).

It has been reported that the begomovirus AC2 can function as a suppressor of transgene induced RNA silencing in a model plant system (Voinnet et al., 1999). Successful generation of transgenic cassava resistant to ACMV was reported through expressing the truncated Rep protein from ACMV in cassava (Fauquet, 2003).

PTGS has been put to use, either by design or by accident, in the development of resistance against the viruses ACMV (Chellappan et al., 2004, Vanderschuren et al., 2007 and Vanitharani et al., 2003), Mungbean yellow mosaic virus (MYMV (Pooggin et al., 2003)), TYLCV (Abhary et al., 2006, Fuentes et al., 2006 and Zrachya et al., 2007), Bean golden mosaic virus (BGMV) (Bonfim et al., 2009).

An advantage of RNA-based approaches is that they might be perceived by regulators as being safer than those that rely on the expression of foreign proteins in plant material consumed by humans and animals. However, there are some potential drawbacks to the use of PTGS-based resistance mechanisms. Broad-based resistance may be difficult to engineer using PTGS because it is homology-dependent and there is therefore likely to be only a small amount of tolerable sequence variation between PTGS inducing transgens and their targeted viruses. In addition, some viruses express proteins that are silencing suppressor (Vanitharani et al., 2005, Bisaro, 2006; Moissiard and Voinnet, 2004 and Sharma, 2008) which could undermine PTGS-based resistance. Such “anti-silencing” determinants include the transcription activator proteins (TrAPs) of ACMV (Voinnet et al., 1999), Indian cassava mosaic virus (ICMV) (Vanitharani et al., 2004), MYMV (Trinks et al., 2005), Tomato yellow leaf curl China virus (van Wezel et al., 2002) and TGMV (Wang et al., 2005); the AC4/C4 genes of Sri Lankan cassava mosaic virus (Vanitharani et al., 2004) and Bhendi yellow vein mosaic virus (BYVMD) (Gopal et al., 2006); the V2 of TYLCV (Zrachya et al., 2007).
and the beta (β) gene encoded by a satellite DNA molecule associated with many monopartite begomoviruses including Tomato leaf curl Java virus (Kon *et al.*, 2007) and BYVMD (Gopal *et al.*, 2006).

### 2.9.2. Resistance due to the expression of non-pathogen derived antiviral agents

#### 2.9.2.1. Virus-induced cell death

Infected plants often have an innate defensive hypersensitive reaction that limits virus movement to the site of infection by inducing the death of infected cells and their neighbours. Such reaction can be artificially induced to provide virus resistance in transgenic plants by the combined action of the barnase and barstar proteins of *Bacillus amyloliquefaciens* (Vanderschuren *et al.*, 2007, Zhang *et al.*, 2005).

Barnase is a ribonuclease (RNase) and barstar its inhibitor. In the absence of virus infection the two transgenes should be expressed at similar levels, resulting in no RNase production. By placing barnase under the control of a viral virion-sense promoter that is activated during virus infection and barstar under the control of a viral complementary-sense promoter that is repressed during virus infection, an infected cell should over-express barnase relative to barstar and die before the infecting virus can replicate and move. While very promising, this and related barnase–barstar based strategies are still only in their early developmental stages.

#### 2.9.2.2. DNA binding proteins

The use of transgenically expressed DNA binding proteins to provide virus resistance relies on the identification of virus sequence-specific binding proteins that will not bind host DNA sequences. The virus Rep is a sequence-specific dsDNA binding protein (Castellano *et al.*, 1999 and Fontes *et al.*, 1992) that recognises and binds to direct repeats in the virion strand origin of replication (v-ori) where it initiates and terminates rolling circle replication (Fontes *et al.*, 1992, Fontes *et al.*, 1994 and Heyraud-Nitschke *et al.*, 1995). This sequence specific activity has been exploited by designing artificial zinc finger proteins with high affinity for the Rep-specific direct repeats in different geminiviruses (Sera and Uranga 2002), with the idea that the artificial zinc finger proteins will competitively block the binding of Rep due to the higher affinity of the artificial zinc finger protein– dsDNA interaction, thereby inhibiting viral replication. The utility of this approach was successfully demonstrated in *Arabidopsis thaliana* against Beet severe curly top virus (BSCTV) (Sera, 2005).
2.9.2.3. GroEL-mediated protection

In a slight twist on more conventional resistance mechanisms, a vector–virus interaction that is essential for efficient virus transmission has been turned into a beneficial interaction for the plant (Akad et al., 2007)). A homologue of GroEL (a chaperonin) produced by endosymbiotic bacteria from the whitefly vector, *Bemisia tabaci* was shown to bind with high affinity to the coat protein of TYLCV (Morin et al., 1999). It was proposed that it may protect the virus from destruction during its passage through the insect’s haemolymph (Morin et al., 1999). This property was used as a tool to trap or capture TYLCV and other plant viruses that interact with GroEL from plant extracts for diagnostic purposes (Akad et al., 2004). GroEL binds a wide range of begomovirus coat proteins it was really a matter of time before the protein was put to the test as a resistance transgene. The *B. tabaci* GroEL gene, expressed in transgenic tomatoes under the control of a phloem-specific promoter, protected the plants from infection with TYLCV (which is phloem limited in tomatoes). Plants infected with TYLCV were either asymptomatic or only mild symptomatic and as expected the GroEL formed complexes with the virus (Akad et al., 2007).

GroEL neither inhibiting virus replication nor preventing virus transmission, the transgene will not strongly select for resistance breaking variants. This ingenious idea of getting a gene that is essential for virus survival in its vector species to protect its host species is an excellent example of how, rather than ensuring the eventual failure of engineered resistance, evolution can be co-opted to ensure its long term durability.

2.9.3. Strategies in the pipeline

2.9.3.1. Peptide aptamers:

A peptide aptamer is a short (20 amino acid long) recombinant protein, constrained within a scaffold protein such as thioredoxin. It strongly binds to a target protein and interferes with its intracellular function (Baines and Colas 2006 and Hoppe-Seyler et al., 2004).

Peptide aptamers were first applied to engineering virus resistance in transgenic *N. benthamiana*, targeting the nucleoprotein (N) of the tospovirus, Tomato spotted wilt virus (TSWV) (Rudolph et al., 2003) and Uhrig et al., 2003). To engineer geminivirus resistance using a similar strategy, Rep specific aptamers were
selected by a yeast two-hybrid screen of a random peptide aptamer library using the N-terminal domain of TGMV Rep as bait (Lopez-Ochoa et al., 2006). Peptides were identified that bind to the Reps of diverse geminiviruses, including CaLCuV, BCTV, East African cassava mosaic virus-Uganda and ACMV–Cameroon (Lopez-Ochoa and Hanley-Bowdoin, 2007), demonstrating their potential in broad-spectrum resistance.

2.9.3.2. InPAct

Most of the expression based resistance mechanisms rely on high-level constitutive expression of recombinant proteins, a novel gene expression system called InPAct (for In Plant Activation) is likely to be part of the ‘next generation’ of inducible transgene expression technologies. The main innovation of the InPAct system is that instead of it depending on promoter transactivation, it directly exploits the extremely specific DNA nicking and joining activities of virus Reps to ensure that gene expression cassettes will only be functional in the presence of these proteins. InPAct system will also be useful for virus-induced expression of non lethal resistance genes for several reasons: (1) constitutive expression of resistance genes is redundant when no when no infection occurs and is likely to add to the metabolic load of the crop, (2) constitutively expressed genes are more likely to be the target of transgene silencing, (3) as mentioned above, the expression of virus proteins such as Rep or movement proteins can cause developmental defects.

2.9.4. Cassava regeneration

2.9.4.1. Primary somatic embryogenesis

Regeneration through somatic embryogenesis is well established and has been used successfully by many workers using different types of explants. The primary Somatic embryogenesis in cassava starts with the induction of primary embryos from leaf or meristem explants (Stamp & Henshaw, 1987a; Szabados et al., 1987; Mroginsky & Scocchi, 1993; Raemakers et al., 1993; Li et al., 1995; Narayanaswamy et al., 1995), zygotic embryos (Stamp & Henshaw, 1982; Konan et al., 1994) or floral tissue (Mukherjee, 1995). In the procedure described by Stamp and Henshaw (1982, 1987) immature leaf lobes were cultured on a medium supplemented with Murashige and Skoog (1962) salts and vitamins, 20 g/l sucrose and 4mg/l 2, 4-D for the induction of embryos. After 15 to 20 days the developed embryos were transferred to a medium with 0.01 mg/l 2, 4-D for maturation of embryos.
Konan et al (1994) demonstrated the development of somatic embryogenesis from seeds of mature cassava cotyledons explants. A two-step medium sequence was developed for efficient embryogenesis. Application of 2, 4-D (4 mg/l) yielded proembryogenic masses which developed into somatic embryos after transfer to a medium containing NAA (0.01 mg /l), BA (0.1 mg /l) and GA3 (0.1 mg /l). Raemakers, (1993) standardized somatic embryogensis in 8 different African cassava cultivars using 2, 4-D using immature leaf lobes. Other investigators showed that in certain genotypes, Dicamba (1–66 mg/l) and Picloram (1–12 mg/l) were superior to 2, 4-D for inducing primary embryogenesis (Ng, 1992; Sudarmonowati& Henshaw, 1993; Taylor & Henshaw, 1993). Mathews et al. (1993) improved maturation of globular embryos in the genotype M. Col1505 by transferring explants after 15 days of culture to a growth regulator-free medium supplemented with 0.5% charcoal, as a result the number of mature embryos increased.

2.9.4.2. Secondary or Cyclic somatic embryogenesis

The common way of induction of primary somatic embryos in cassava is using leaf lobes or seed derived cotyledons culturing on 2, 4-D containing medium. Somatic embryos can be used as starting explants for a new cycle. A novel method of cyclic embryogenesis of cassva on solidified medium was described (Raemakers et al., 1993b). Cyclic embryos, originating from embryos, could be obtained in both liquid and on solid medium. The same group later reported that the production of embryos in liquid medium was distinctly higher, faster and more synchronized than on solid medium. The production of single embryos was achieved by pressing starting embryos through a fine meshed sieve, indicating that embryos can be produced from a piece of tissue with a restricted number of cells. The shoot conversion rate of embryos from liquid medium was comparable with that of embryos from solid medium (Raemakers, 1993a).

In cassava 2,4D induced both primary and secondary embryogensis and NAA only secondary embryogenesis (Raemakers, 1993). The wounding was not obligatory but it increased the number of embryos formed. Not only, 2, 4-D, Picloram and Dicamba, but also NAA had the capacity to induce secondary embryogenesis. In general more mature embryos were produced in NAA supplemented medium than in 2, 4-D, supplemented medium. Furthermore, the development of NAA induced embryos was faster than with 2, 4- D, Dicamba or Picloram (Sofiari, 1996). Groll et al (2001) reported the secondary somatic embryogensis of cassava on picloram supplemented media. He also reported that (2002) addition of abscicic acid and charcoal to the somatic embryo culture medium improved the regeneration efficiency of cassava somatic embryos.
2.9.4.3. Friable Embryogenic Callus (FEC) and suspension culture

A less organized or more indirect type of somatic embryogenesis in cassava was developed by Taylor et al. (1996). With continuous selection, organized embryogenic tissue, cultured on a medium supplemented with Gresshoff and Doy (1972) salts and vitamins and 10 mg/l picloram (GD2) converted gradually into a less organized tissue. This tissue consisted of a friable callus-like mass of pro-embryos. The cells in the friable embryogenic callus (FEC) were continuously in a state where they break away from group control and because of that they were not organized into a unified structure. This type of embryogenesis can be described as indirect or unorganized somatic embryogenesis. Another difference was the basic origin of the new embryos. In FEC the origin of the new embryos are claimed to be at the surface of the embryogenic units (Taylor et al., 1996) and in primary and secondary somatic embryogenesis from multiple cells from internal tissue (Stamp & Henshaw, 1987c; Raemakers et al., 1995b). Pure friable embryogenic callus (FEC) lines can be maintained with a three weeks subculture regime on GD2 medium for more than 2 years.

Suspension cultures were initiated by culturing FEC in Schenk and Hildebrandt (1972) medium supplemented with 60 g/l sucrose and 10 mg/l picloram (SH6). FEC develops into mature embryos after culture on maturation medium (Sofiari, 1996). The mature embryos were multiplied by secondary somatic embryogenesis at high frequency and after desiccation the mature embryos germinated into plants. In vitro the plants are morphological similar to the original genotype and almost all plants survived transfer to the greenhouse.

2.9.4.4. Shoot organogenesis

Shoot formation through organogenesis was achieved from stem callus (Tilquin 1979) and mesophyll protoplast cultures (Shahin and Shepard 1980). However, these results were not reproducible. Li et al (1998) demonstrated that plants could be regenerated at high frequency by inducing shoot primordial on explants derived from cotyledons of cassava somatic embryos. After a passage on elongation medium, the regenerated shoots were easily rooted in hormone free medium and could be successfully transplanted to soil. This method widens the scope of in vitro regeneration modes of cassava, and is also compatible with Agrobacterium-mediated transformation.

In the organogenesis system, development of shoot primordial is induced directly on cytokinin-containing media from cotyledon explants of germinating somatic embryos. A cyclic system in which
secondary somatic embryos are induced on cotyledon explants from maturing somatic embryos is used to provide a constant source of regeneration competent explant material.

Using the shoot-organogenesis-based regeneration method, Shoot primordia could be induced on pieces of cotyledons derived from cycling somatic embryos on a medium containing the combination of 1.0 mg/l BA and 0.5 mg/l IBA (Li, 1998). Callus appeared on the cut surface after 3 days of culture. The first buds appeared 7 days later and were either formed directly from the cut surface or were connected with the callus. After 20 days of induction in the dark shoots were isolated from the explants and cultured either on the same medium or on a medium for rooting in the light. After 60 days the plants had a length of 10 to 20 cm (Li et al., 1995, 1996). Zhang et al (2000) reported that adding silver nitrate to the regeneration medium improved the regeneration efficiency and reduced callus formation from cotyledonary explants. It was found that the highest shoot organogenesis rates were obtained by supplementing the medium with 2 and 1mg/L AgNO3.

2.9.4.5. Protoplast culture

There is only one report of shoot regeneration from protoplasts of cassava (Shahin and Shepard 1980). These authors used well expanded leaves for protoplast isolation. However, this could not be repeated by others (Nzoghe 1989; Anthony et al. 1995; Sofiari 1996). Anthony et al. (1995) improved plating efficiency by culturing protoplasts of cassava with short glass rods in ammonium-free Murashige and Skoog (1992) medium.

The first successful establishment of protoplast culture in cassava was reported by Sofiari et al (1998) using friable embryogenic callus as a starting material. Protoplasts were isolated from friable embryogenic callus (FEC) and from suspensions derived from FEC of cassava genotype TMS60444. Their results showed that protoplasts plated at a density of 105–106/ml in a medium supplemented with 0.5 mg/l α-naphthaleneacetic acid and 1 mg/l zeatin began dividing after 3 days, and after 30 days this resulted in an absolute plating efficiency as high as 2.5%. After 2 months of culture, 60% of the developed calli were highly friable and in appearance identical to the original FEC. The protoplast derived FEC was first purified through two rounds of selection of 3 weeks each before being cultured for regeneration of plants. This was done by culturing the protoplast-derived FEC for 11 weeks on maturation medium, yielding a maximum of 184 organized embryos per 10,000 initially cultured protoplasts. Most of the organized embryos were torpedo shaped and matured after they had been isolated from the calli and transferred to fresh medium.
Mature embryos were multiplied by secondary somatic embryogenesis at high efficiency (>90%) on a medium supplemented with 8 mg/l 2, 4- dichlorophenoxyacetic acid. About 30% of the mature secondary somatic embryos developed into shoots after transfer to a medium supplemented with 1 mg/l N6-benzylaminopurine (BAP). Shoots rooted readily on a medium without BAP.

2.9.5. Cassava genetic transformation

Several reports are there on genetic transformation studies on cassava (Table 3). Somatic embryogenesis forms the basis for all current transformation methods of cassava. Cyclic embryogenesis is the most widely used method of de novo plant regeneration of cassava invitro and provides constant source for material for plant transformation. Plant regeneration from somatic embryos can be established via either direct germination (Stamp et al., 1987; Mathews, 1993; Li et al., 1995; Taylor et al., 1996, 2001) or shoot organogenesis (Li, 1998; Zhang, 2001). Currently shoot organogenesis from somatic cotyledons (Li, 1996; Zhang, 2000a, 2000b) and embryogenic suspension from derived from friable embryogenic callus (FEC) (Schopke, 1996; Gonzales, 1998) are the two most frequently used systems for production of transgenic cassava plants. Other methods like electroporation of protoplast culture were also under trial.

2.9.5.1. Methods of genetic transformation

Traditional breeding of cassava is difficult due to irregular flowering and low fertility as well as to low seed set and germination rates of the plants, and attempts to improve the protein content of cassava roots have so far been unsuccessful. Advances in plant genetic engineering now provide an alternative to traditional breeding in improving cassava. Several reports are available on the transformation of cassava (Li et al., 1996; Raemakers et al., 1996; Schöpke et al., 1996; Gonzalez et al., 1998; Zhang et al., 2000a, b). Since 1996 three methods of gene transfer have proven successful in producing transgenic cassava plants; particle bombardment (Schöpke et al., 1996, Raemakers et al., 1996), electroporation (Sofiari, 1996) and Agrobacterium-mediated transformation (Li et al., 1996; Gonzalez et al., 1998).

With both particle bombardment and Agrobacterium-mediated transformation, friable embryogenic callus has been successfully used as target tissue. An efficient and reproducible method was developed for Agrobacterium-mediated transformation of embryogenic suspension cultures of cassava by Schreuder et al. (2001).
They used the Agrobacterium strain LBA4404 containing the super binary vector pTOK233 and used kanamycin for selection of transformants. Protocols for cassava transformation were reported simultaneously by two different research groups in 1996. Li et al. used Agrobacterium-mediated transformation of somatic cotyledons to then regenerate transgenic shoots by organogenesis. Schöpke et al., however, performed microparticle bombardment of embryogenic suspension-derived tissues and then regenerated transgenic plantlets by embryo maturation. In the latter system, transformation relied on the implementation of a protocol to generate totipotent cell clusters, known as friable embryogenic callus (FEC).

Agrobacterium-mediated transformation of FEC, a combination of the two original systems, has subsequently emerged as the most efficient and widely used strategy to produce transgenic cassava. This combined protocol is superior as, first, by using FEC there is a reduced risk of generating chimeric plants compared to procedures using organized tissues, such as cotyledons. Second, selection (usually antibiotic resistance) of FEC results in fewer nontransformed plantlets being regenerated (i.e., escapes) compared to shoot organogenesis, FEC and shoot organogenesis systems are compatible with both biolistic and Agrobacterium mediated gene transfer and with several selectable markers. Antibiotic selection using Hygromycin (Zhang, 2000) and positive selection using mannose (Zhang, 2000) can be used for both shoot organogenesis and FEC based methods, and paramomycin, kanamycin, or visual selection using firefly luciferase as screenable marker or a combination of antibiotic selection and luciferase screening (Schopke, 1996; Gonzales, 1998; Raemakers, 2001; Munyikawa, 1998) can also be used to produce transgenic plants from FEC.

2.9.5.2. Marker genes

A range of selectable and visual marker genes have been tested and developed for use in cassava transformation systems. The nptII gene, which imparts resistance to the amylloglycoside antibiotics, is used routinely by several laboratories, with selection for successful transformation events possible by culturing tissues on medium containing kanamycin, paramomycin or geneticin. Efficacy of the hygromycin resistance gene (hpt) has also been demonstrated, but is reported to suppress the frequency of plant recovery compared to transgenic tissues selected by expression of nptII (Raemakers et al., 2001; Schreuder et al., 2001; Hankoua, 2003).
Use of the bar gene to impart resistance to the herbicide phosphinothricin acetyltransferase is utilized both as a gene of agronomic interest and as a selection system. In order to develop protocols for the recovery of transgenic plants without antibiotic or herbicide resistance genes, use of the Escherichia coli phosphomannose isomerase (pmi) gene was used to transform FEC and cotyledon explants and procedures for the effective recovery of transgenic tissues and plants using this positive selection system have been developed (Zhang and Pounti-Kaerlas, 2000; Zhang et al., 2000a). In addition, all three visual marker systems, GUS, luciferase and GFP, have been successfully established and are routinely employed as tools for developing transgenic systems in cassava and investigating transgene expression patterns (Li et al., 1996; Raemakers et al., 1996; Schopke et al., 1996).

2.9.5.3. Explants for genetic transformation

2.9.5.3.1. The use of secondary somatic embryogenesis for plant transformation

Evidence for the production of transgenic embryos and plants was first provided in 1994 (Sarria et al., 1995). Sarria et al transformed cassava somatic embryos with a wild strain of Agrobacterium in 1995. Since the transgenic plants were containing oncogenes it was of no agronomic value. Bombardment of embryos with GUS gene resulted in 1% transformation efficiency (Schopke et al., 2000). But the repeated subculture of transformed embryos resulted in the loss of transformed cells. Most probably this can be explained by the fact that only epidermal cells will be transformed and it has been shown that in secondary somatic embryogenesis of cassava the embryos originate from deeper cell layers (Stamp, 1987c; Raemakers et al., 1995b).

2.9.5.3.2. The use of friable embryogenic callus for plant transformation

Schopke et al 1996 established a protocol for the introduction of DNA into embryogenic suspension derived tissues of cassava via microparticle bombardment. The plasmid DNA used for bombardment contained a gene encoding npt II and a gene encoding uidA. Selection of bombardarded tissues with paramomycin resulted in the establishment of putative transgenic embryogenic calli in most of these calli, GUS was detected and further molecular analysis of regenerated plants from these calli confirmed the stable integration of bombardarded DNA into cassava genome.

Taylor et al (2001) first reported the production and recovery of transgenic cassava plant through microbombardment of friable embryogenic suspension culture. Significant numbers of transgenic plants
containing transgene for putative resistance to cassava mosaic virus and in addition to visual marker genes have been regenerated. Transgenic plants from three African cultivars of cassava were recovered after particle bombardment of embryogenic suspension culture. Agrobacterium-mediated transformation of friable embryogenic calli (FEC) is the most widely used method to generate transgenic cassava plants.

However, this approach has proven to be time-consuming and can lead to changes in the morphology and quality of FEC, influencing regeneration capacity and plant health.

Zhang et al. (2003) transformed the cassava plants with the artificial storage protein (ASP1) gene using Agrobacterium-mediated transformation method. They used embryogenic cell suspension as explants for transformation and hygromycin as selection agent to screen the transformants.

Transgenic cassava expressing a hairpin dsRNA homologous to the sequence including the bidirectional promoter and CR of African cassava mosaic virus were first reported by Zhang et al (2007) through FEC transformation. The same group earlier reported that transgenic cassava expressing the antisense RNAs of ACMV Rep, TrAP and REn could resist ACMV infection via post transcriptional gene silencing (Zhang et al, 2005). They used RNAi vector pRNAI-DPRO and used cassava suspension cultures for transformation using Agrobacterium strain LBA4404.

Bull et al., in 2009 demonstrated a comprehensive, reliable and improved protocol, which takes a short duration using Agrobacterium-mediated transformation of FEC. In this method FEC is cocultivated with Agrobacterium directly on the propagation medium and adopted the extensive use of plastic mesh for easy and frequent transfer of material to new media. This minimizes stress to the FEC cultures and permits a finely balanced control of nutrients, hormones and antibiotics. A stepwise increase in antibiotic concentration for selection is also used after cocultivation with Agrobacterium to mature the transformed FEC before regeneration.

2.9.5.3.3. The use of protoplast culture for plant transformation

Electroporation of protoplasts is another method which will become applicable in the near future. Both protoplasts isolated of secondary somatic embryos and from friable embryogenic callus were electroporated with pJIT64. This resulted with protoplasts isolated from secondary somatic embryos in transgenic callus. Unfortunately, as described before, protoplasts isolated of somatic embryos do not regenerate into plants. The experiments with protoplasts of FEC are ongoing. However, the transient LUC
activity of these protoplasts was higher than of protoplasts isolated from secondary somatic embryos. Six weeks after electroporation distinct LUC spots were observed which increased in LUC activity in the subsequent assays. Although it is not a definite proof, the increase in LUC activity points towards stable transformation. The efficiency of the procedure is about one stable transformation event per 107 electroporated protoplasts. It is assumed that, as was the case with particle bombardment, chemical selection will increase the efficiency of the procedure (Raemaker, unpublished results).

2.9.5.3.4. The use of somatic cotyledons for transformation

Li et al (1996) reported the use of somatic cotyledons for geneic engineering cassava. They used the Agrobacterium strain LBA4404 carrying the binary vector pTOK233 carrying an intron interrupted uidA gene. Four days of cocultivation gave the optimum combination of high transformation rate and controllable bacterial growth.

A novel protocol, based on biolistics and regeneration via organogenesis, was developed for genetic transformation of cassava (Manihot esculenta Crantz) by Zhang et al 2000). The effect of different parameters for particle bombardment efficiency, including the amount of DNA used, the flying distance of the projectiles and the pre- and post-plasmolysis time of the target tissue, was evaluated and the conditions were partially optimised. Stably transformed cassava plants of MCol22 and TMS60444 were produced using the partially optimised conditions and two different vectors constructs carrying the hpt gene as the selectable marker.
Table 1. Summary of Geminiviruses resistance strategies in crop plant

<table>
<thead>
<tr>
<th>Source of transgenic sequence</th>
<th>Transgene</th>
<th>Plant in which resistance was shown</th>
<th>Virus against which resistance was shown</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Bean golden mosaic virus</td>
<td>Rep gene mutants</td>
<td>Tobacco suspension cells</td>
<td>Bean golden mosaic</td>
<td>Hanson and Maxwell (1999)</td>
</tr>
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<td>Virus Type</td>
<td>Mutant Type</td>
<td>Host Plant 1</td>
<td>Virus Type</td>
<td>Reference</td>
</tr>
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</tr>
<tr>
<td>Maize streak virus</td>
<td>rep gene mutants and truncated rep gene mutants</td>
<td>Digitaria sanguinalis</td>
<td>Maize streak virus</td>
<td>Shepherd et al. (2007)</td>
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<tr>
<td>Maize streak virus</td>
<td>Truncated rep gene mutant</td>
<td>Maize</td>
<td>Maize streak virus</td>
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</tr>
</tbody>
</table>

### 2. Movement protein genes (MP)

<table>
<thead>
<tr>
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<th>MP Mutant Type</th>
<th>Host Plant 1</th>
<th>Virus Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato mottle virus</td>
<td>Mutated mp gene</td>
<td>Nicotiana tabacum</td>
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<td>Bean dwarf mosaic virus</td>
<td>Mutated mp gene</td>
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### 3. Gene silencing

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<tbody>
<tr>
<td>African cassava mosaic virus</td>
<td>DNA-A bidirectional promoter</td>
<td>Cassava</td>
<td>African cassava mosaic virus</td>
<td>Vanderschuren et al. (2007)</td>
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<td>African cassava mosaic virus</td>
<td>Chemically synthesized siRNA to REP mRNA</td>
<td>Nicotiana tabacum</td>
<td>African cassava mosaic virus</td>
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<tr>
<td>Mungbean yellow mosaic virus</td>
<td>DNA-A bidirectional promoter</td>
<td>Blackgram</td>
<td>Mungo yellow mosaic virus</td>
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<td>RNA Type</td>
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<td>Tomato yellow leaf curl virus</td>
<td>Hairpin RNA derived from REP gene</td>
<td>Tomato and tobacco</td>
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<td>Abhary et al. (2006)</td>
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<td><strong>4. Antisense</strong></td>
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<td>Cotton leaf curl Kokhran virus</td>
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<td>Nicotiana tabacum</td>
<td>Cotton leaf curl Kokhran virus</td>
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<tr>
<td>African cassava mosaic virus</td>
<td>REP, TrAP and REn</td>
<td>Cassava</td>
<td>African cassava mosaic virus</td>
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</table>

**Expression of non-pathogen derived antiviral agents**

1. Virus-induced cell death

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antiviral Agents</th>
<th>Host</th>
<th>Virus</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Bacillus amyloliquefaciens</td>
<td>BARNASE and BARSTAR genes</td>
<td>Cassava</td>
<td>African cassava mosaic virus</td>
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</table>

2. DNA binding proteins

<table>
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<th>Virus</th>
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<tbody>
<tr>
<td>Artificially produced</td>
<td>Sequence encoding Artificial Zinc Finger Protein (binds to viral origin of replication)</td>
<td>Arabidopsis thaliana</td>
<td>Beet severe curly top virus</td>
<td>Sera. (2005)</td>
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3. GroEL

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<th>Host</th>
<th>Virus</th>
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<tr>
<td>Endosymbiotic bacteria from Bemisia tabaci</td>
<td>GroEL gene (binds to viral Coat Protein)</td>
<td>Tomato</td>
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4. Peptide Aptamers

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<td>Random peptide aptamer library</td>
<td>Recombinant peptide aptamers (bind to viral Rep protein)</td>
<td>Nicotiana tabacum protoplasts</td>
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<td>Meristem culture</td>
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<td>TMS60444</td>
<td>leaf</td>
<td>Somatic embryogenesis</td>
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<td>Somatic embryogenesis</td>
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<td>Secondary somatic embryos</td>
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<td>Taylor et al. 1996,Sofiari et al., 1996</td>
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<td>Explants</td>
<td>Method of transformation</td>
<td>Gene</td>
<td>Reference</td>
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<td>MCol22</td>
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<td>Agrobacterium mediated</td>
<td>Gus</td>
<td>Li et al., 1996; González et al., 1998;</td>
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<tr>
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<td>Raemakers et al., 2001</td>
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<td>Particle bombardment</td>
<td>Antisense AC1 gene</td>
<td>Taylor et al., 2004</td>
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CHAPTER III
MATERIALS AND METHODS

3.1. Standardization of regeneration protocol in cassava variety, H-226

3.1.1. Establishment of in vitro mother plants

The cassava cultivar, H-226 cuttings with 5-8 nodes were obtained from mature greenhouse grown plants. The nodal cuttings were initially washed with tap water and then washed two times with distilled water. Then shoots were cut with single node and surface sterilized with 70% ethanol for 1 min followed by 2.5% Sodium hypochlorite solution for 5 minutes. Then the explants were washed 6 times in sterile water and dried in sterilized Whatmann filter paper. These Nodal cuttings with a single node (2-3 cm long) were then cultured in propylene capped glass jars (diameter 6 cm; height 12 cm) with 50 ml of Murashige and Skoog (MS) basal salts, MS vitamins supplement with 2% sucrose and solidified with 4 g/l phytigel, pH 5.7. The cultures were incubated in growth chamber with 28 ºC and photoperiod of 14 h. After one month of incubation, the nodal cuttings with proliferated shoots were transferred to fresh medium for 30 days. Then the immature leaf lobes were excised from the shoot and used for callus induction. The remaining shoots portion were cut with single node and cultured in fresh medium for further multiplication.

3.1.2. Embryogenic callus induction in cassava

Immature leaf lobes of 0.5 -1 cm in size were tried as explants for embryogenic callus initiation in cassava. The leaf lobes were collected from 20 to 25 days old in vitro grown mother plants. Two different growth hormones viz., 2,4-D (2,4,6,8,10,12,14,16 mg/l) and Picloram were experimented in different concentration (3,6,9,12,15,18 mg/l) to test their efficacy in inducing embryogenic calli (Table 4). Young leaf lobes which were just sprouted or 2nd or 3rd leaves from the shoot meristem were taken for inoculation and fifty leaf lobes were kept per petriplate (size 90 x 15 mm, Axygen). The adaxial surface of the leaf lobes were kept in contact with the somatic embryo induction medium (SEIM) to prevent non embryogenic calli proliferation. The inoculated cultures were sealed with parafilm and kept in continuous dark at 26 ºC. Calli proliferation starts from the cut end of the leaf lobes in 10 days. After 20 days, the leaf lobes with proliferated calli is transferred to fresh medium and incubated in dark at 26 ºC. Two to three sub culturing is done to obtain the embryogenic structures on the surface of calli.
3.1.3. Somatic embryo maturation

After development of somatic embryos on callus surface, somatic embryos with the adjoining calli will be separated from the callus and they were transferred to somatic embryo maturation medium (SEMM). The SEMM medium consists of MS salts and B5 vitamins with 3% maltose and cytokinins. Different combinations and concentrations of cytokinins were tested for their efficacy in embryo maturation (Table 4). The somatic embryos were sub cultured every 20 days interval till matured green somatic cotyledons were developed.

3.1.4. Secondary (cyclic) somatic embryogenesis

To induce secondary somatic embryogenesis, the green coloured mature cotyledonary stage somatic embryos were harvested at every 20 days interval and chopped to small size of 0.5 cm and incubated in SEIM at 26 °C in dark. Five media compositions were tested to find out their efficacy on secondary embryo development on somatic cotyledon explants (Table 4). The mature cotyledonary stage somatic embryos on culturing in SEIM medium gave raise to globular stage embryos and then follow the embryogenic developmental pathway. The torpedo stage somatic embryos were transferred to SEMM to form the cotyledonary stage embryo and the process repeated at regular interval.

3.1.5. Plant regeneration

Cotyledonary stage somatic embryos either from the primary somatic embryogenesis or secondary somatic embryogenesis were transferred to Regeneration medium (RM) and incubated at 26 C with 16 h photo period for plant regeneration (Table 4). Regeneration medium contains MS salts, B5 vitamin, 0.1 mg/l BAP, 0.02 mg/l NAA, 3% maltose, 4 g/l Phytage. After three weeks, the somatic embryos were transferred to Regeneration medium supplemented with different concentrations of GA3 for shoot elongation (Table 4). Regenerating cultures were incubated in 16 h photo period at 26°C and sub cultured at every 20 days interval until proper shoot and root primordial emerges. Then 1-3 cm size plantlets were transferred to MS basal medium containing 2% sucrose and 4 g/l phytagel for further plant growth.

3.1.6. Hardening

The fully developed plantlets with two to three leaves were transferred into sterilized soil mixture (1:1:1 ratio of sand: clay soil: coir pith) in small pots under controlled conditions and then transferred to transgenic greenhouse.
3.2. Isolation and characterization of Rep gene of CMV from Tamil Nadu.

3.2.1. Source of virus and Maintenance (CMV) of inoculum

Virus Infected tapioca setts were collected from different locations of TamilNadu and Kerala (Table 5). Young leaves showing severe curling and mosaic symptoms were used for total genomic DNA extraction.

3.2.2. DNA extraction

Total DNA was extracted from the second or third leaf from the plant apex using CTAB method of plant genomic DNA extraction (Lodhi et al., 1994) (Appendix 1). The DNA concentration was quantified by 0.8% agarose gel electrophoresis and used as template for PCR amplification.

3.2.3. Isolation and characterization of ICMV and SLCMV Rep gene

3.2.3.1. 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) and SLCMV (Accession No. AJ890225, AJ579307, AJ607394, AJ890227 and AJ890228) were downloaded from NCBI Genebank.

The ICMV and SLCMV Rep gene sequences were multiple aligned separated using ClustalX programme and conserved regions were found. Primers were designed for the amplification of 1058 bp full length Rep gene manually. The ICMV Rep gene amplified using the designed Forward primer (ICMV Rep FP: 5'TTAGCTGCTCTGTGTGTGACC3'), and Reverse Primer (ICMV REPRP: 5'ATGACACCTCTCTAAGCGCTTTC3'). The SLCMV Rep gene was amplified using the designed Forward primer (SLCMV REP FP: 5'TTAGCTGCTCTGTGTGTGACC3') and Reverse Primer (SLCMV REP RP: 5' ATG AGA AACCCA CGA TTCAG 3').

3.2.3.2. PCR analysis of Rep gene

The DNA extracted from infected cassava leaves 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 fidelity DNA polymerase.
<table>
<thead>
<tr>
<th>S.NO</th>
<th>District</th>
<th>Location</th>
<th>Variety</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Salem</td>
<td>Athur</td>
<td>H226</td>
</tr>
<tr>
<td>2.</td>
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<td>Athur</td>
<td>Mulluvadi</td>
</tr>
<tr>
<td>3.</td>
<td>Salem</td>
<td>Athur</td>
<td>H226</td>
</tr>
<tr>
<td>4.</td>
<td>Salem</td>
<td>Athur</td>
<td>Kumkuma rose</td>
</tr>
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</tr>
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<td>6.</td>
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</tr>
<tr>
<td>7.</td>
<td>Coimbatore</td>
<td>Ondiputhur</td>
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<td>Kalvadipudir</td>
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<tr>
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<td>Erode</td>
<td>Udayampalaym</td>
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<tr>
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<td>Bhavani</td>
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</tr>
<tr>
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<td>Kallakurichi</td>
<td>Unknown</td>
</tr>
<tr>
<td>21.</td>
<td>Namakkal</td>
<td>Thothannur</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
(Finnzyme, Finland) and 13.9 µl sterilized double distilled water. Amplification was performed in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., Watertown, USA).

3.2.3.2.1. Temperature profile and PCR cycles used for gene specific primer

- Initial Denaturation
  - 94 °C for 5 min
- Denaturation
  - 94 °C for 1 min
- Primer annealing
  - 58 °C for 1.0 min
- Primer extension
  - 72 °C for 2 min
- Final extension
  - 72 °C for 15 min

Amplified PCR products were electrophoresised through 1 % (w/v) agarose gel.

3.2.3.3. Cloning of PCR amplified DNA in CloneJet™ (pJET1.2/blunt) vector (Fermentas, USA)

pJET1.2/blunt is a linearized cloning vector (Appendix 2), which accepts inserts from 6 bp to 10 kb. Blunt-end PCR products generated by proofreading DNA polymerases can be directly ligated in just 5 min with the pJET1.2/blunt cloning vector. This vector contains a lethal gene (eco47IR) which is disrupted by ligation of a DNA insert into the cloning site. As a result only cells with recombinant plasmids are able to propagate; the self ligated molecules will express the lethal gene and will be killed. As a result, only recombinant clones containing the insert appear on culture plates. Therefore, blue/white screening is not required.

3.2.3.4. Ligation

The ligation reaction for blunt end cloning was set up in a final volume of 20 µl with 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 (5u/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 22°C for 5 min. Two microliter of the ligation mixture was directly used for bacterial transformation.
3.2.3.5. Bacterial transformation

3.2.3.5.1. Preparation of competent cells

Single colony of DH5α strain was inoculated in 3 ml LB broth (without antibiotic) and incubated at 37°C overnight. One ml of the overnight grown culture was inoculated into 30 ml LB broth and grown at 37°C in a rotary shaker at 200 rpm, till 0.4-0.5 OD600 growth. Cell suspension was maintained at 0°C for 20 min, and then the cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C followed by resuspension in 10 ml of sterile ice cold 50 mM CaCl₂ and incubated again on ice for 20 min. The cell suspension was centrifuged at 5000 rpm for 10 min at 4°C. The pellet was resuspended in 2 ml of sterile ice cold 100 mM CaCl₂ and stored as aliquots of 100 µl at 4°C, for immediate use. For long-term storage, 15% glycerol was added and the cells were preserved at -70°C.

3.2.3.5.2. 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.

3.2.3.6. Selection of transformants

3.2.3.6.1. Colony PCR

The recombinant clones were screened using two sets of primers, gene specific as well as pJET vector specific forward and reverse primer. Reactions were performed using a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., Watertown, USA) with single colony of DH5α. Temperature profiles described in section 3.3.1.4 and 3.3.1.5 were used for amplification. After amplification, 10 µl of the product was used for electrophoresis analysis on a 1.0% agarose gel. The reaction mixture for both PCR is as following; 2.0 µl of 10X Taq buffer, 2.0 µl of dNTP mix (2 mM each) 0.4 µl of Forward and reverse primer (10 mM), 0.3µl of Taq DNA polymerase (5 u/µl) (Genie, India) and the final PCR reaction volume was made up to 20 µl using nuclease free water. 1.2 µl of 25 mM MgCl₂ was added in pJET vector specific PCR
reaction alone for more specificity. Colonies which gives positive amplification of 1.0 kb with both pJET and gene specific primers were selected as recombinant ones. The non recombinant colonies will not give 1.0 kb amplification and will give 100bp amplification with vector specific primer which will be the amplification of the intervening vector sequences at the ligation site of pJET1.2/Blunt vector.

3.2.3.6.2. Plasmid isolation and restriction analysis

Single colony from LB agar (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract, 15 g/l agar, pH 7.2) with Ampicillin (100 mg/l) was first short streaked in LB agar plate with Ampicillin (100mg/l) and kept at 37°C for overnight. A patch of bacterial culture was taken from this plate and inoculated in 5 ml LB broth containing Ampicillin (100 mg/l) and grown overnight at 37°C with 200 rpm shaking. The plasmid DNA was isolated by Sigma Minipreps plasmid isolation kit (Catalog # A1700; Sigma, USA). Restriction analysis of plasmid DNA isolated from recombinant colonies was performed in a reaction volume of 20 µl containing 500 ng of plasmid DNA, 10 U of BglII using appropriate buffer (O buffer) at 37 °C for 1 h. Digested products were analyzed on 0.8% agarose gel.

3.2.3.7. Sequencing and phylogenic analysis of cloned isolates of ICMV and SLCMV

The recombinant colonies were sent for sequencing (Genei Bangalore). The sequencing result was analyzed through NCBI BLAST search. The BLAST programme (Altschul et al., 1990) was used to identify related sequences available from the GenBank databases as well as to get the homology percentage. The phylogenetic relations and multiple sequence alignments were done to find out the sequence similarity and genetic divergence of the isolated ICMV and SLCMV clones using Neighbor-Joining method using the PHYLIP program NEIGHBOR (Felsenstein 2005). The analyzed clones were further submitted in NCBI GenBank, USA.

3.3. Construction of gene silencing vectors for cassava mosaic disease control

3.3.1 Construction of RNAi vector for control of ICMV

3.3.1.1. Cloning of partial ICMV Rep gene into RNAi intermediate vector, pHANNIBAL

The RNAi intermediate vector, pHANNIBAL (Appendix 3) was obtained from CSIRO plant industry, Australia. This vector (with bacterial ampicillin resistance) is designed for directional insertion of PCR
products on either side of the PDK intron. A conserved sequence of size 440 bp from the 5’ region of the Rep gene sequence of ICMV was identified after multiple sequence alignment using BioEdit software and primers were designed to amplify 440 bp Rep gene of ICMV covering 112 – 540 nt region of Rep gene (Table 6). The restriction enzymes, Xhol and KpnI were appended with sense forward and reverse primer and BamHI and HindIII were appended with antisense forward and reverse primer of ICMV respectively. The PCR amplification was done as per the procedure given in section 3.2.4.1. except that the annealing temperature was kept at 60°C and the cloned full length sequence was used as PCR template.

3.3.1.2. Cloning of sense ICMV Rep gene into pHANNIBAL

The 440bp sense Rep gene of ICMV was PCR amplified and resolved in Agarose gel and eluted from the gel using GenElute™ Gel Extraction kit (Sigma, USA). The eluted insert DNA was digested with Xhol and KpnI restriction enzyme. The pHANNIBAL vector was linearized by digestion with Xhol and KpnI followed by gel elution. The sense ICMV rep gene insert was ligated with linearized pHANNIBAL and transformed into E.coli strain, DH5α. The cells were plated in LB agar plate containing 100 mg/l Ampicillin. The recombinant clones were identified by PCR screening the colonies using the sense Rep gene primers as well as by restriction digestion with Xhol and KpnI. One of the recombinant clones containing the cloned sense ICMV Rep gene was further used for cloning the Antisense of the Rep gene.

3.3.1.3. Cloning the antisense ICMV Rep gene into pHANNIBAL clone containing the sense Rep gene.

The 440bp anti-sense Rep gene of ICMV was PCR amplified and resolved in Agarose gel and eluted from the gel using GenElute™ Gel Extraction kit (Sigma, USA). The eluted insert DNA was digested with BamHI and HindIII restriction enzyme. The pHANNIBAL vector cloned with sense Rep gene of ICMV was linearized by digestion with BamHI and HindIII followed by gel elution. Then the linearized vector and insert was ligated and transformed into E.coli strain, DH5α. The cells were plated in LB agar plate containing 100 mg/l Ampicillin. The recombinant clones were identified by PCR screening the colonies using the Ocs terminator specific reverse primer (Ocs RP: 5’CCAACGATTTGTCGTCACTG 3’) and Rep gene reverse primer (Rep RP: 5’ATACGGAGGTGGTGGTTGTT 3’). Then the clones were further confirmed by restriction digestion with BamHI and HindIII. The complete hairpin RNAi gene cassette was released by digestion with NotI restriction enzyme.
3.3.1.4. Cloning of RNAi-Rep gene cassette into plant transformation vector, pART-27

The pART27 (Appendix 4) plant transformation binary vector was obtained from CSIRO Plant Industry, Australia. The pART27 vector contain nptII gene as plant selectable marker. The cloned RNAi gene cassette was released from pHANNIBAL (cloned with sense and antisense of the Rep gene) by NotI enzyme digestion and cloned into the NotI site of binary vector, pART27. The recombinant clones were identified by blue/white selection in presence of 40 mg/l X-gal and 100 mg/l Spectinomycin as well as by restriction digestion with NotI enzyme to release the hairpin cassette. The pART27 vector with RNAi-ICMV Rep gene cassette was designated as pICR1.

3.3.2. Construction of RNAi vector for control of SLCMV

A partial 440 bp region from the 5’ end of the full length Rep (starting from 112 bp to 540 bp) gene of SLCMV was amplified using SLCMV Rep gene specific primers (Table 6). The construction of vector was done following the procedure described in section 3.6.1. The constructed pART27 vector with RNAi-SLCMV Rep gene cassette was designated as pSCR1.

3.3.3. Cloning of RNAi-Rep gene cassette into plant transformation vector containing GUS gene and Hygromycin selectable marker.

The RNAi-Rep gene cassette of ICMV and SLCMV was cloned into MCS (Multiple Cloning Site) of pCAMBIA vectors 1300 and 1305.2, both the vector have hph gene as plant selectable marker. The RNAi cassette of ICMV and SLCMV was initially cloned into the NotI site of pBLUESCRIPT (SK+) vector (Appendix 5) and then the insert was released with SalI and SacI restriction digestion and cloned into the pCAMBIA1300 and pCAMBIA1305.2 (Appendix 6). The recombinant clones were identified by restriction analysis with SalI and SacI enzyme. The recombinant plasmids were named pICR2 & pICR 3, pSCR2 & pSCR 3 for ICMV and SLCMV respectively.
Table 6. Primers designed for amplification of sense and Antisense of Rep gene of CMV used for RNAi vector construction

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<th>Sno</th>
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<th>Primer (ICMV )</th>
<th>Total length</th>
<th>Primer Position</th>
</tr>
</thead>
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<td>1</td>
<td>ICMV Sense FP</td>
<td>5'CGCCTCGAG CCCACAAACCCAAAATTCA 3' XhoI</td>
<td>28</td>
<td>112 –131</td>
</tr>
<tr>
<td>2</td>
<td>ICMV Sense RP</td>
<td>5' CCC <strong>GGTACC</strong> ATACGGAGGTGGTGGTGTGTT 3' KpnI</td>
<td>28</td>
<td>521- 540</td>
</tr>
<tr>
<td>3</td>
<td>ICMV Antisense FP</td>
<td>5' CCC <strong>GGATCC</strong> CCCACAAACCCAAAATTCA 3' BamHI</td>
<td>28</td>
<td>112 –131</td>
</tr>
<tr>
<td>4</td>
<td>ICMV Antisense RP</td>
<td>5' CCC <strong>AAGCTT</strong> ATACGGAGGTGGTGGTGTGTT 3' HindIII</td>
<td>28</td>
<td>521- 540</td>
</tr>
<tr>
<td>5</td>
<td>SLCMV Sense FP</td>
<td>5' CCCCTCGAG CCCACAAACCCAAAATTCA 3' XhoI</td>
<td>28</td>
<td>112 –131</td>
</tr>
<tr>
<td>6</td>
<td>SLCMV Sense RP</td>
<td>5' CCCGGTACC ATACGGAGGTGGTGGTGTGTT 3' KpnI</td>
<td>28</td>
<td>521- 540</td>
</tr>
<tr>
<td>7</td>
<td>SLCMV Antisense FP</td>
<td>5' CGC <strong>GGATCC</strong> CCCACAAACCCAAAATTCA 3' BamHI</td>
<td>28</td>
<td>112 –131</td>
</tr>
<tr>
<td>8</td>
<td>SLCMV Antisense RP</td>
<td>5' CCAAGCTT ATACGGAGGTGGTGGTGTGTT 3' HindIII</td>
<td>28</td>
<td>521- 540</td>
</tr>
</tbody>
</table>
3.4. Genetic transformation of cassava for CMD resistance

3.4.1. Genetic transformation of cassava variety H-226 for SLCMV resistance

3.4.1.1. Agrobacterium strain

A donor *E. coli* strain DH5α harbouring the recombinant plasmids, pSCR2 and (SLCMV specific RNAi vector) and an *E. coli* conjugative helper strain, DH5α harbouring pRK2013 were grown separately on LB agar plates containing Spectinomycin (100mg/l) and Kanamycin (50 mg/l) respectively. The *Agrobacterium* recipient strain, LBA4404 was grown on a YEP agar (10 g/l peptone, 5 g/l NaCl, 10 g/l yeast extract, 15 g/l agar, pH 7.0) plate containing Rifampicin 10mg/l. The RNAi-Rep vectors were mobilized into Agrobacterium strain LAB4404 using triparental method (Appendix).

3.4.1.2. Plant material

Young immature leaf lobes of of 0.5 to 1 cm in size cassava just near to the apical meristem and green coloured individual mature somatic cotyledons of 1.0 cm in size separated from cluster of germinated somatic embryos (This stage is obtained 20 to 30 days after transfer of somatic embryo into somatic embryo maturation medium) were used for cocultivation. These explants were precultured on SEIM for 2 days in dark at 28°C.

3.4.1.3. Cocultivation

The *Agrobacterium* strain LBA4404 harboring the pSCR2 vector was inoculated in 50 ml culture and shaken overnight at 250 rpm in LB medium at 26°C until the OD560 was 1.0. The *Agrobacterium* culture was then pelleted at 4000 rpm, supernatant discarded and the pellet was resuspended in AAM broth supplemented with 100 µm acetosyringone. Explants (both leaflobes and green cotyledons) were dipped in *Agrobacterium* solution for 10 min and thoroughly blot dried using sterile Whatmann filter paper. After Agrobacterium infection, the explants were transferred to Cocultivation medium containing MS salts, B5 vitamin, 12 mg/l Picloram, 30 g/l Maltose, 4 g/l Phytagel, 100 µm Acetosyringone, and pH-5.8 and incubated for 48 h in darkness at 26°C. After cocultivation, explants were washed twice with sterile distilled water and once with ½ MS basal salts and vitamins containing 300 mg/l Cefotaxime. Explants were then
3.4.1.4. Selection and plant regeneration

After 48 hours of co-cultivation, the treated explants were kept in somatic embryo induction medium (SEIM) with hygromycin 30 mg/l and cefatoxime 300 mg/l. The cultures were incubated in dark at 26 °C. Two rounds of selection were done to obtain transformed tissue. The subculturing was done at 20 days interval. The somatic embryos developed at the end of second subculture in SEIM were transferred to SEMM. After 20 days on maturation media, mature somatic embryos were transferred to regeneration medium (MS basal salts, MS vitamin, 3% sucrose, 0.4% Phytagel) containing plant growth regulators 0.1 mg/L of BAP, 0.02 mg/L of α-naphthalene acetic acid (NAA) and 0.2 mg/L GA₃. The somatic embryos cultured in regeneration medium were incubated under light with 16/8 photoperiod. Subculture was done every two weeks in fresh regeneration medium and incubated under light. Two to three subcultures were done for shoot regeneration.

3.4.2. Genetic transformation of cassava for ICMV resistance

Similarly the explants were co-cultivated using *Agrobacterium strain LAB4404* harbouring pICR1 in separate experiment. The co-cultivation and selection procedures were done as described in section except that the selection agent used here was Kanamycin at 100mg/l concentration.

3.5. Molecular analysis of putative transgenic lines of cassava

3.5.1. Genomic DNA extraction for PCR

Small leaf bits (2-3 cm) were ground in a 1.5 ml Microfuge tube containing 300 µl of extraction buffer (200 mM Tris-HCl pH 7.5, 200 mM NaCl, 25 mM EDTA and 0.5 % SDS) and acid-washed sand using a pestle. The homogenate was centrifuged at 12,000 rpm for 10 min. Equal volume of isopropanol was added to the supernatant and kept at - 20 °C for 20- 30 min. The crude DNA was pelleted by centrifugation at 12,000 rpm for 10 min; pellets were air dried at room temp and dissolved in 30 µl of 0.1X TE buffer (1.0 mM Tris-HCl pH 8.0 and 0.1 mM EDTA pH 8.0; Proebksi *et al.*, 1997). For each PCR reaction, 100 ng of genomic DNA was used as template.
3.5.2. PCR analysis of putative transgenic cassava shoots transformed with SLCMV specific gene constructs.

The PCR analysis was carried out using 100 ng of genomic DNA in a 20 µl reaction mixture containing 2.0 µl of 10X PCR buffer (50 mM Tris-HCl pH 8.8, 50 mM KCl and 1.5 mM MgCl2), 200 µM of each dNTPs, 1 µl of each primer (forward and reverse) and 2 units of Taq DNA polymerase. The primer sequences used for amplification of hph gene are: Forward primer (H1 - 5’GATCTCCAATCTGCGGGATC3’) and Reverse primer (H3- 5’ACTCACCGCGACGTCTGTCG3’). The hygromycin sequence in total DNA was amplified in a PTC-100 minicycler (MJ Research, USA) with following temperature conditions, pre-incubation period at 94 °C for 3 min, leading to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and synthesis at 72°C for 1 min, followed by extension at 72°C for 5 min. Amplified PCR product (10 µl) was subjected to electrophoresis on a 0.8% agarose gel and visualized under UV light. The SLCMV Rep gene in putative transgenic plants were amplified as described in section 3.3.1.4 using the SLCMV Rep gene specific primers

3.5.3. PCR analysis of putative transgenic cassava shoots transformed with ICMV specific gene constructs.

The genomic DNA from putative transgenic shoots of ICMV transgenic plants were extracted as described in section 3.5.1. The primer sequences used for amplification of nptII and gene was as follows: Forward primer: 5’ CTGATGCCTCCAGAT and Reverse primer: 5’ AGAGGCTATTCGGCTATGACT. The PCR conditions for nptII was as following initial denaturation 94 °C for 5 minute, denaturation 94 °C for 1 minute, primer annealing 60 °C for 40 seconds, primer extension 72 °C for 45 seconds; 72 °C for 1 minute and final extension 72 °C for 10 minutes respectively. The steps 2 to 4 repeated 30 times. Amplified PCR product (10 µl) was subjected to electrophoresis on a 0.8% agarose gel and visualized under UV light. The ICMV Rep gene in putative transgenic plants were amplified as described in section 3.3.1.4 using the ICMV Rep gene specific primers
CHAPTER IV
EXPERIMENTAL RESULTS

In this study an attempt was done towards genetic transformation of cassava resistant to CMD through RNA interference approach. As cassava is recalcitrant to genetic transformation, well standardized regeneration protocol is required. The first part of the study was done to develop somatic embryogenesis in elite genotype of Indian cassava variety, H-226, and then the subsequent multiplication of the embryogenic tissue by secondary (cyclic) somatic embryogenesis followed by plant regeneration. The second aspect deals with cloning and characterization of Indian and Sri Lankan cassava mosaic virus in Tamilnadu followed by construction of RNAi vectors for control of CMD. The final part deals with the transformation and recovery of transgenic plants transformed with the developed RNAi constructs.

4.1. Standardization of regeneration protocol in cassava variety H-226

4.1.1. Shoot tip culture to establish *in vitro* mother plant culture

The single nodal cuttings of field grown mother plant produced fresh leaves after 2-3 weeks of inoculation in MS basal medium (Plate 1A). Most of the nodal cuttings showed normal growth with well developed leaves. However, some of the nodal cuttings showed stunted growth with small leaves. The young leaf lobes were taken for primary somatic embryogenesis from nodal cuttings (3-4 weeks) which are showing normal growth (Plate 1B). After taking the leaf lobes, the shoot were cut with individual nodes and placed on MS basal medium for obtaining more leaf lobes for embryogenic callus induction.

4.1.2. Primary somatic embryogenesis in cassava

Immature leaf lobes were cultured in somatic embryo induction medium. After 10 days, immature leaf lobes (Plate 1D) became swollen and after 20 days, friable unorganized calli formed from the cut end of the leaf lobes (Plate 1E). The leaf lobes with calli were transferred to fresh somatic embryo induction medium twice for callus proliferation. After 40 days in the somatic embryo induction medium, some of the proliferating calli turned smooth on the calli surface which later showed development of globular somatic embryos (Plate 1F, G). Only the proliferating calli with globular somatic embryos were further transferred to fresh somatic embryo induction medium for development of somatic embryos into heart, torpedo, and
Plate 1. Primary somatic embryogenesis of Cassava variety H-226

A. Shoots containing 4-5 nodes
B. Portion of shoot with single node
C. In vitro plants after 30 days
D. Immature leaf lobes on SEIM
E. Leaf lobes after 10 days on SEIM
F. Appearance of friable calli after 20 days
G. Microscopic view of embryogenic calli
H. Appearance of globular stage embryo
I. Heart shaped stage
J. Torpedo stage embryos
K. Germinated embryos on SEIM
L. Matured cotyledons after 15 days on SEIM
cotyledonary stages. The development of globular somatic embryo into cotyledonary stages takes two weeks of culturing in somatic embryo induction medium (Plate 1H, 1I, 1J & 1K).

It was observed that adaxial surface of the leaf lobes placed in contact with the medium gave rise to embryogenic calli from the cut ends, whereas, abaxial surface of leaf lobes in contact with the medium gave rise to watery non embryogenic calli from cut ends of the leaf lobes. The frequency of primary somatic embryo formation on leaf lobe explants was maximum in (54.25%) medium containing 4 mg/l 2, 4-D (Table 7). In another separate experiment with different concentration of Picloram for callus induction, maximum embryogenic frequency of 58.00% was obtained in (SEIM 4) at 12 mg/l of Picloram (Table 7). Attempt was made further to increase the embryogenic calli frequency by modifying the other media constituents like vitamins and gelling agents with 4 mg/l 2, 4-D or 12 mg/l of Picloram in the somatic embryo induction medium. This study reported a somatic embryo induction frequency which varied from 52.67% to a maximum of 66.77% (Table 8). Highest somatic embryo induction of 66.77% was obtained in SEIM5, which consists of MS minerals, B5 vitamins, 12 mg/l Picloram, 3% maltose and 4% Phytagel.

In addition to using the leaf lobes for callus induction, the shoot tip after removing the leaf lobes were also used as explant for somatic embryo induction. Even though shoot tip explants showed calli proliferation, but the development of somatic embryos were rare. Apart from leaf lobes and shoot tip, fully expanded leaves were also tried for embryogenic callus induction. Like shoot tip, the fully expanded leaf showed calli proliferation without somatic embryo formation.

4.1.3. Secondary or cyclic somatic embryogenesis in cassava

When primary somatic embryos at cotyledon stage (Plate 2A) were separated from primary explants and transferred onto fresh somatic embryo induction medium, direct formation of secondary globular embryos obtained without an intervening callus phase on the green cotyledonary surface (Plate 2B). The globular embryos emerged directly on the surface of somatic cotyledons after 10 days on somatic embryo induction medium. Later small swellings appeared on the apical region of the somatic embryos (Plate 2C) and new embryos developed from these initials (Plate 2D). The secondary embryos followed all the stages of somatic embryogenic development as in the case of primary embryogenesis. It was also observed that some of the globular somatic embryos developed into cotyledonary stage without passing through the torpedo stage. Among the five different secondary embryogenic induction media tested, maximum
Table 7. Influence of different concentrations of Auxins on primary somatic embryo induction from immature leaf lobes of cassava variety, H226

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (mg/l)</th>
<th>Frequency of somatic embryogenesis (%)</th>
<th>Concentration of Picloram (mg/l)</th>
<th>Frequency of somatic embryogenesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>41.75 (32.56)c</td>
<td>3</td>
<td>33.00 (35.05)d</td>
</tr>
<tr>
<td>4</td>
<td>54.25 (46.72)a</td>
<td>6</td>
<td>35.25 (36.41)d</td>
</tr>
<tr>
<td>6</td>
<td>45.75 (41.26)b</td>
<td>9</td>
<td>39.25 (38.78)c</td>
</tr>
<tr>
<td>8</td>
<td>47.50 (40.83)b</td>
<td>12</td>
<td>58.00 (49.60)a</td>
</tr>
<tr>
<td>10</td>
<td>45.50 (40.10)c</td>
<td>15</td>
<td>48.75 (44.28)b</td>
</tr>
<tr>
<td>12</td>
<td>37.15 (28.12)d</td>
<td>18</td>
<td>22.25 (19.22)e</td>
</tr>
<tr>
<td>14</td>
<td>22.25 (16.17)e</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>8.75 (6.74)e</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data is a mean of 3 replications with 50 explants per replicate for 2, 4, D and 4 replications with 50 Explants per replicate for Picloram

Table 8. Effects of different media composition on Embryogenic calli induction from immature leaf lobes of cassava variety, H226

<table>
<thead>
<tr>
<th>Somatic Embryo Induction Media (SEIM)</th>
<th>Vitamins</th>
<th>Gelling agent (%)</th>
<th>Auxin (mg/l)</th>
<th>Frequency of embryogenic calli(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEIM1</td>
<td>MS Vitamins</td>
<td>0.4% Phytgel</td>
<td>2,4-D (4)</td>
<td>55.00 (47.34)d</td>
</tr>
<tr>
<td>SEIM2</td>
<td>MS Vitamins</td>
<td>Phytgel</td>
<td>Picloram (12)</td>
<td>52.67 (46.53)d</td>
</tr>
<tr>
<td>SEIM3</td>
<td>MS Vitamins</td>
<td>0.6% Agarose</td>
<td>2,4-D (4)</td>
<td>63.67 (52.93)a</td>
</tr>
<tr>
<td>SEIM4</td>
<td>MS Vitamins</td>
<td>0.6% Agarose</td>
<td>Picloram (12)</td>
<td>59.67 (50.57)b</td>
</tr>
<tr>
<td>SEIM5</td>
<td>B5 Vitamins</td>
<td>0.4% Phytgel</td>
<td>2,4-D (4)</td>
<td>64.00 (53.14)a</td>
</tr>
<tr>
<td>SEIM6</td>
<td>B5 Vitamins</td>
<td>0.6% Agarose</td>
<td>Picloram (12)</td>
<td>66.67 (54.74)a</td>
</tr>
<tr>
<td>SEIM7</td>
<td>B5 Vitamins</td>
<td>0.6% Agarose</td>
<td>2,4-D (4)</td>
<td>55.67 (48.25)c</td>
</tr>
<tr>
<td>SEIM8</td>
<td>B5 Vitamins</td>
<td>0.6% Agarose</td>
<td>Picloram (12)</td>
<td>53.67 (47.10)d</td>
</tr>
</tbody>
</table>

The data is a mean of 3 replications with 50 explants per replicate
Plate 2. Cyclic somatic embryogenic induction and regeneration from somatic cotyledons

A. Somatic cotyledons on SEIM

B. Globular embryos on cotyledons after 10 days on SEIM

C. Formation of secondary embryos

D. Proliferated secondary embryos after 30 days on SEIM

E. Embryo maturation

F. Regenerated plants
proliferation of secondary embryos were obtained from SEIM1 (80.00%)(Table 9). The cotyledonary stage embryos are harvested and sub cultured at every 20 days interval to produce more somatic embryos.

4.1.4. Somatic embryo maturation and regeneration

Ten different maturation media were tested to promote maturation of somatic embryos and regeneration of plants. Somatic embryos with their adjoining callus (45 to 60 days old) were transferred to somatic embryo maturation medium. After two weeks in maturation medium, green, folios structures developed first (Plate 3A) and after 25 to 30 days, plantlets with cotyledon like leaves emerged (Plate 3B). Among different maturation medium tested, SEMM 2 with 0.2 mg/l BAP and 0.01 mg/l NAA gave a maximum of 80.00% embryo maturation (Table 10). The shoot elongation and formation of normal leaves and shoot occurred when it is transferred to regeneration medium containing gibberellins (Plate 3C). Among the five different regeneration media tested, maximum regeneration efficiency and normal shoot and root development occurred in RGM 4 (85.25%) which contains 0.2 mg/l GA3 in addition to 0.2 mg/l BAP and 0.01 mg/l NAA (Table 11). The embryos are transferred to fresh regeneration medium at every 20 days interval. The well rooted plantlets (Plate 3D) were transferred to green house (Plate 3E).

4.2. Isolation and characterization of Rep gene of ICMV and SLCMV from Tamil Nadu.

Total genomic DNA was isolated using CTAB method from 44 samples of infected cassava leaves taken from five districts of Tamil Nadu and one sample from Kerala and was used for PCR amplification of Rep gene of ICMV and SLCMV (Plate 4A). Using the designed gene specific primers, the expected full length ICMV (Plate 4B) as well as partial SLCMV Rep gene of size 1058 bp and 750 bp respectively were obtained by PCR amplification (Plate 4C). 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 and 750 bp for SLCMV from the recombinant colonies of ICMV and SLCMV respectively (Plate 4D & 4E). In case of recombinant clones with ICMV Rep gene, the clones were further confirmed by restriction digestion with BglII to release the 1058 bp DNA fragment (Plate 4F).

DNA sequencing confirmed the cloning of Rep gene of ICMV and SLCMV 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 and SLCMV sequences by Gene Bank (Table 12).
Table 9. Secondary somatic embryogenic in cassava somatic cotyledons with different concentrations of Picloram

<table>
<thead>
<tr>
<th>Cyclic somatic embryogenesis medium (CSEM)</th>
<th>Picloram (mg/l)</th>
<th>Frequency of secondary somatic embryogenesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSEM1</td>
<td>3</td>
<td>61.00(52.41)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CSEM2</td>
<td>6</td>
<td>61.00(51.36)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CSEM3</td>
<td>9</td>
<td>78.50(62.41)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CSEM4</td>
<td>12</td>
<td>80.00(63.49)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CSEM5</td>
<td>15</td>
<td>60.00(51.23)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The data is a mean of 4 replications with 10 explants per replicate.
Plate 3. Regeneration of Cassava variety H-226

A. Matured cotyledons after 15 days on SEMM
B. Formation of green foliose like structures
C. Formation of cotyledonary leaf
D. Normal leaf and shoot development
E. Shoot and root elongation
F. Plants after six months
G. Hardening of invitro plants
H. Establishment of regenerated plants in soil
Table 10. Effect of different concentrations of BAP and NAA on Somatic embryo maturation in cassava

<table>
<thead>
<tr>
<th>Somatic Embryo Maturation Media (SEMM)</th>
<th>BAP (mg/l)</th>
<th>NAA (mg/l)</th>
<th>Frequency of somatic embryo germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEMM1</td>
<td>0.10</td>
<td>0.01</td>
<td>16.67(23.98)\textsuperscript{h}</td>
</tr>
<tr>
<td>SEMM2</td>
<td>0.10</td>
<td>0.02</td>
<td>80.00(66.03)\textsuperscript{a}</td>
</tr>
<tr>
<td>SEMM3</td>
<td>0.20</td>
<td>0.01</td>
<td>69.33(56.44)\textsuperscript{p}</td>
</tr>
<tr>
<td>SEMM4</td>
<td>0.20</td>
<td>0.01</td>
<td>70.67(57.22)\textsuperscript{p}</td>
</tr>
<tr>
<td>SEMM5</td>
<td>0.30</td>
<td>0.01</td>
<td>61.00(51.36)\textsuperscript{c}</td>
</tr>
<tr>
<td>SEMM6</td>
<td>0.30</td>
<td>0.02</td>
<td>48.00(43.85)\textsuperscript{f}</td>
</tr>
<tr>
<td>SEMM7</td>
<td>0.40</td>
<td>0.01</td>
<td>58.00(49.61)\textsuperscript{d}</td>
</tr>
<tr>
<td>SEMM8</td>
<td>0.40</td>
<td>0.02</td>
<td>50.00(45.00)\textsuperscript{e}</td>
</tr>
<tr>
<td>SEMM9</td>
<td>0.50</td>
<td>0.02</td>
<td>25.67(30.42)\textsuperscript{g}</td>
</tr>
<tr>
<td>SEMM10</td>
<td>1.00</td>
<td>0.02</td>
<td>15.00(22.23)\textsuperscript{h}</td>
</tr>
</tbody>
</table>

The data is a mean of 3 replications with 10 explants per replicate.

Table 11. Effect of different concentrations of GA\textsubscript{3} on somatic embryo regeneration in cassava

<table>
<thead>
<tr>
<th>Regeneration media (RGM)</th>
<th>BAP+NAA (mg/l)</th>
<th>GA\textsubscript{3} (mg/l)</th>
<th>Frequency of regeneration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGM1</td>
<td>0.1 mg/l+0.02 mg/l</td>
<td>0.05</td>
<td>36.75(37.28)\textsuperscript{d}</td>
</tr>
<tr>
<td>RGM2</td>
<td>0.10</td>
<td>0.10</td>
<td>37.00(37.43)\textsuperscript{d}</td>
</tr>
<tr>
<td>RGM3</td>
<td>0.15</td>
<td>0.20</td>
<td>56.50(48.74)\textsuperscript{c}</td>
</tr>
<tr>
<td>RGM4</td>
<td>0.15</td>
<td>0.20</td>
<td>85.25(67.47)\textsuperscript{a}</td>
</tr>
<tr>
<td>RGM5</td>
<td>0.25</td>
<td>0.25</td>
<td>76.25(60.88)\textsuperscript{b}</td>
</tr>
<tr>
<td>RGM6</td>
<td>0.30</td>
<td>0.30</td>
<td>76.10(60.02)\textsuperscript{b}</td>
</tr>
</tbody>
</table>

The data is a mean of 4 replications with 10 explants per replicate.
Plate 4. Screening of ICMV and SLCMV Rep gene from symptomatic leaves of cassava

4A. PCR amplification of ICINV/Rep gene
Lane 1: 100bp ladder 2: ICINV/Rep gene amplification from infected sample

4B. Confirmation of ICINV/Rep gene cloned in pCLONEJET by colony PCR
Lane 1: 100bp ladder 2-5: Positive clones

4C. Release of ICINV/Rep gene from pCLONEJET by restriction digests (lane 2: Undigested; lane 3: Restriction digestion with BglII)

4D. PCR amplification of SLG1MV/Rep gene
Lane 1: 100bp ladder 2-5: Samples tested for the presence of SLG1MV

4E. Confirmation of SLG1MV/Rep gene cloned in pCLONEJET by colony PCR
Lane 1: 100bp ladder 2-5: Positive clones
<table>
<thead>
<tr>
<th>Indian cassava mosaic virus (ICMV)</th>
<th>Isolate</th>
<th>Accession number by NCBI</th>
<th>Srilankan cassava mosaic virus (SLCMV)</th>
<th>Isolate</th>
<th>Accession number given by NCBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thaliyur</td>
<td>HQ415765</td>
<td></td>
<td>Athur 1</td>
<td>HQ415776</td>
<td></td>
</tr>
<tr>
<td>Sangagiri</td>
<td>HQ415766</td>
<td></td>
<td>Athur 2</td>
<td>HQ415777</td>
<td></td>
</tr>
<tr>
<td>Athur</td>
<td>HQ415767</td>
<td></td>
<td>Athur 3</td>
<td>HQ415778</td>
<td></td>
</tr>
<tr>
<td>Thandamuthur</td>
<td>HQ415768</td>
<td></td>
<td>Athur 4</td>
<td>HQ415779</td>
<td></td>
</tr>
<tr>
<td>Ondiputhur</td>
<td>HQ415769</td>
<td></td>
<td>Ondiputhur</td>
<td>HQ415780</td>
<td></td>
</tr>
<tr>
<td>Pollachi</td>
<td>HQ415770</td>
<td></td>
<td>Thandamuthur</td>
<td>HQ415781</td>
<td></td>
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<tr>
<td>Kerala</td>
<td>HQ415771</td>
<td></td>
<td>Sangagiri</td>
<td>HQ415782</td>
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<tr>
<td>Avinashi</td>
<td>HQ415772</td>
<td></td>
<td>Kerala</td>
<td>HQ415783</td>
<td></td>
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<tr>
<td>Erode</td>
<td>HQ415773</td>
<td></td>
<td>Satyamangalam</td>
<td>HQ415784</td>
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<tr>
<td>Bhavani</td>
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<td>Udayampalayam</td>
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<tr>
<td>Namakkal</td>
<td>HQ415775</td>
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<td>Bhavani</td>
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<td></td>
<td></td>
<td></td>
<td>Avinashi</td>
<td>HQ415787</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Namakkal</td>
<td>HQ415788</td>
<td></td>
</tr>
</tbody>
</table>
4.2.2. Field distribution and molecular variability of ICMV and SLCMV isolates from Tamilnadu

Cassava infected samples from five major cassava growing districts of Tamil Nadu was analyzed for the presence of ICMV and SLCMV infection using PCR (Table 13). Mixed infection of ICMV and SLCMV were observed in some of the samples from all the five district of Tamil Nadu as these samples showed PCR amplification for both ICMV and SLCMV. This result shows that mixed infection is common in all the five major cassava growing district of Tamil Nadu. According to the present study more than 60% of the cassava leaf samples from Salem District were infected with SLCMV (Figure 2A), whereas, 20 % of the samples showed infection by ICMV and remaining 20% of the samples showed mixed infection. In case of samples from Coimbatore district, 54 % of samples showed ICMV infection, whereas, 23% showed SLCMV infection and remaining 23% showed mixed infection. Cassava samples from Erode district showed equal proportion of ICMV and SLCMV with 40% infection, whereas 20% of samples showed mixed infection. This distribution pie chart thus shows that there is a recent invasion by ICMV of an area which is originally colonized by SLCMV (Figure 2A, 2B, and 2C).

4.2.3. Phylogenic analysis of ICMV Rep gene isolates from Tamil Nadu

ICMV Rep sequences were trimmed to contain only the regions of high quality sequences. Sequence variations were scored and distance matrixes were generated by the Neighbor-Joining method using the PHYLIP program NEIGHBOR (Felsenstein, 2005). Dendrograms were generated by cluster analysis using the unweighted pair group method using the arithmetic average (UPGMA) (Rohlf 2000), and presented by TREEWIEW (Page, 2000). To validate the phylogenetic trees Bootstrap values were generated with 100 pseudo replicates generated by SEQBOOT and trees were drawn for all pseudo replicates with DNA, NJ and UPGMA program consense was used to make a consenses tree. The bootstrap values higher than 70% were placed on the dendrograms (Figure 3).

The phylogenic analysis showed that the cloned isolates of ICMV Rep gene splits into three main branches (clusters). These clusters are formed by well defined groups with the Salem and Coimbatore ICMV Rep gene isolates falling into one group with 70% bootstrap support. The second group is formed by ICMV Maharashtra and Kerala isolate (with 94% bootstrap support) and the third group formed by the Erode and Namakkal isolates with 97% bootstrap support. The branch distance between the ICMV Rep gene isolates from Namakkal & Avinashi, Kerala & Maharashtra and Athur & Ondiputhur were found to be less showing a less sequence divergence between these isolates. This also emphasize that these paired
Table 13. Identification of CMD causing virus through PCR screening of symptomatic cassava leaf sample from Tamil Nadu

<table>
<thead>
<tr>
<th>District</th>
<th>Location</th>
<th>Variety</th>
<th>Total number of samples analyzed</th>
<th>Virus species present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No of samples positive for SLCMV</td>
<td>No of samples positive for ICMV</td>
</tr>
<tr>
<td>1 Salem</td>
<td>Attur</td>
<td>H226</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2 Salem</td>
<td>Attur</td>
<td>MVD1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>3 Salem</td>
<td>Attur</td>
<td>Kumkuma Rose</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>4 Attur</td>
<td>Unknown</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5 Salem</td>
<td>Unknown</td>
<td>1</td>
<td>1</td>
<td>Nil</td>
</tr>
<tr>
<td>6 Sangagiri</td>
<td>Unknown</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>7 Vilupuram</td>
<td>Kallakurichi</td>
<td>Unknown</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>8 Erode</td>
<td>Sathyamangalam</td>
<td>Unknown</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>9 Erode</td>
<td>Vedachinnamur</td>
<td>Unknown</td>
<td>1</td>
<td>Nil</td>
</tr>
<tr>
<td>10 Erode</td>
<td>Sindhanaicknur</td>
<td>Unknown</td>
<td>1</td>
<td>Nil</td>
</tr>
<tr>
<td>11 Erode</td>
<td>Kasipalayam</td>
<td>Unknown</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>12 Erode</td>
<td>Sempulichampalayam</td>
<td>Unknown</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>13 Erode</td>
<td>Muniappanpalayam</td>
<td>Unknown</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>14 Erode</td>
<td>Kalvadipudur</td>
<td>Unknown</td>
<td>1</td>
<td>Nil</td>
</tr>
<tr>
<td>15 Erode</td>
<td>Udayampalayam</td>
<td>Unknown</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>16 Erode</td>
<td>Bhavani</td>
<td>Unknown</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>17 Erode</td>
<td>Thuthannur</td>
<td>Unknown</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>18 Coimbatore</td>
<td>Coimbatore</td>
<td>Unknown</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>19 Coimbatore</td>
<td>Thondamuthur</td>
<td>Unknown</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>20 Coimbatore</td>
<td>Pollachi</td>
<td>Unknown</td>
<td>1</td>
<td>Nil</td>
</tr>
<tr>
<td>21 Kerala state</td>
<td>Calicut</td>
<td>Unknown</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>22 Kerala state</td>
<td>Calicut</td>
<td>Unknown</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 2. Field distribution of CMD in major cassava districts of Tamil Nadu

CMV field distribution in Salem
- ICMV: 60%
- SLCMV: 20%
- BOTH: 20%

CMV field distribution in Coimbatore
- ICMV: 54%
- SLCMV: 23%
- BOTH: 23%

CMV field distribution in Erode
- ICMV: 40%
- SLCMV: 40%
- BOTH: 20%
Figure 3. Phylogenetic tree of ICMV Rep gene isolated from Tamil Nadu
sequences are relatively closer compared to the other ICMV Rep gene isolates in the phylogenetic tree. The Rep gene isolates from Coimbatore showed a less diverging pattern revealing that the ICMV Rep isolates from areas of the same district showed less divergence. This was similar in the case of ICMV Rep isolates from Erode. However the ICMV Rep gene isolates from Pollachi and Ondiputhur (from Coimbatore district) showed a slight divergence from the other Coimbatore ICMV Rep gene isolates. The relative closeness between ICMV Rep isolates from neighboring places like Erode and Namakkal shows that there can be a little movement of the ICMV through infected planting materials. The north Indian isolate, ICMV-Maharashtra and ICMV-Kerala isolates are relatively well diverged from the other ICMV Rep gene isolates. The out groups SLCMV and MYMV Rep gene isolates from Tamil Nadu are well separated from the ICMV Rep isolates (Figure 3).

4.2.4. Phylogenetic analysis of SLCMV Rep gene isolates from Tamil Nadu

The Rep genes of SLCMV were compared keeping Mungbean Yellow Mosaic Virus (MYMV) from Tamil Nadu as out group which showed 65% identity with SLCMV. The phylogenetic analysis of SLCMV Rep gene sequences showed that the cloned isolates of SLCMV Rep gene splits into three main branches (clusters). However, these clusters formed by the SLCMV Rep gene isolates did not show clustering based on geographical separations as which happened in the case of ICMV (Figure 4). In each clusters there were one representation of isolate from each of the five districts surveyed. The out group MYMV Rep gene isolates from Tamil Nadu is well separated from the SLCMV Rep isolates.

4.3. Construction of RNAi-Rep gene silencing vectors for control of ICMV and SLCMV

4.3.1. 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 (Plate 5A). 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 Xhol/Kpn1 site. The recombinant clones were confirmed by colony PCR using Rep gene specific forward
Figure 4. Phylogenic tree of SLCMV Rep gene isolated from Tamil Nadu
and reverse primer which amplified an expected size of 440 bp (plate 5B). Then the pHANNIBAL cloned with sense Rep gene was used for cloning the antisense Rep gene in the HindIII/BamHI 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 antisense of the Rep gene were further confirmed by XhoI/HindIII and KpnI/BamHI double digestion, which released the 1.2 kbp fragment (Plate 5C). 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 NotI restriction digestion (Plate 5D).

4.3.2. Cloning of Rep-RNAi cassettes in pART27 vector

The released 3.8 kb RNAi Rep gene cassette fragment from pHANNIBAL was cloned into the NotI 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 (plate 5E). After identifying the recombinant clones in colony PCR, this was further confirmed by restriction digestion with NotI as well as with BamHI and SalI (Plate 5F). The NotI digestion released two fragments; the 3.8 kbp RNAi-Rep gene cassette and the vector backbone of 11.6 kb. The double digestion with BamHI and SalI 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 (Figure 5A). 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.

4.3.3. Construction of RNAi-Rep vector for control of SLCMV

Similarly, RNAi vector for SLCMV was made as described in section 4.3.1 and 4.3.2. The SLCMV Rep- RNAi vector was designated as pSCR1 (Figure 5B). The binary vectors pSCR1 were mobilized into the Agrobacterium strain LBA4404 by Triparental mating method. The presence of pICR1 and pSCR1 in Agrobacterium strain LBA4404 was confirmed by colony PCR and by restriction digestion of recombinant plasmid from back transformed colonies using KpnI and BamHI (Plate 6A and Plate 6B).


- Panel B: Confirmation of IC3V Rho gene insertion in pHW101 by digestion. Lane 1: 1 Kb Ladder, 2-5: Restriction enzyme digestion


- Panel D: Release of Rho-RNAa cassette from plasmid pART27. Lane 1: 1 Kb Ladder, 2: Digestion of plasmid pART27 alone, 3: Digestion of plasmid pART27 + IC3V Rho gene


Figure 5. Physical map of RNAi vectors, pICR1 and pSCRI

pICR1
15400 bp

pSCRI
15400 bp
Plate 8. Confirmation of pICR1 vector in Agrobacterium strain LBA4434

6A. Confirmation of pICR1 plasmid by cloning/PCR:
Lane 1. 1 kb ladder 2. Negative Control 3-5 Agrobacterium positive clones (Transgenic)

6B. Bac transformed clones digested with
KpnI/BamHI
Lane 1. 1 kb ladder 2-3 Bac transformed clones shown digested with KpnI/BamHI
4.3.4. 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 and SLCMV were cloned into pCAMBIA1305.2 vector that contain the hph gene and Gus gene. Since NotI restriction site is not available in the pCAMBIA vector, the RNAi gene cassettes were first sub cloned into NotI site of pBLUESCRIPT (SK+) vector. Then the 3.8 kb RNAi gene cassette was released from the pBLUESCRIPT by double digestion with SalI/SacI (Plate 7A). The 3.8 kb Rep-RNAi fragment was then cloned into SalI and SacI site of pCAMBIA 1305.2. The recombinant clones containing the Rep-RNAi cassette in pCAMBIA 1305.2 was confirmed by double digestion with SalI and Sal to release the 3.8 kb cassette (Plate 7B). The RNAi binary vectors of ICMV and SLCMV with this pCAMBIA1305.2 were named pICR2 and pSCR2 respectively (Figures 6A and 6B).

4.4. Genetic transformation of cassava variety H-226 for SLCMV resistance

In order to obtain transgenic cassava resistant to SLCMV, the Agrobacterium strain LBA4404 containing the RNAi- SLCMV Rep gene cassette in pSCR2 with hph as gene selectable marker was used for genetic transformation of cassava. Two explants immature leaf lobes (Plate 8A) and somatic cotyledons (Plate 9A) were tried. Out of 3000 immature leaf lobes co cultivated 106 calli survived two rounds of Hygromycin (30 mg/l) selection (Plate 8B, 8C, 8D & 8E). The resistant embryos when transferred to somatic embryo maturation medium resulted in germination of 64 embryos (Plate 8G) and further to regeneration medium resulted in regeneration of 40 lines to give a transformation efficiency of 1.6 % (Table 14). Out of 500 somatic cotyledons (derived from primary somatic embryos) cocultivated 24 survived two rounds of Hygromycin (30 mg/l) selection. The resistant embryos when transferred to somatic embryo maturation medium resulted in germination of 18 embryos. The germinated embryos when transferred to regeneration medium resulted in regeneration of 6 shoots giving a transformation efficiency of 1.2% (Table 14). In total, out of the 48 transgenic shoots developed two shoots developed well and are hardened in greenhouse (Plate 8K & 8L).

4.4.2. Genetic transformation of cassava variety H-226 for ICMV resistance

Agrobacterium strain LBA4404 harbouring pICR1 in with nptII gene is available as plant selectable marker was used for genetic transformation of cassava. Two explants, immature leaf lobes and somatic cotyledons were used for Agrobacterium- mediated transformation. Out of 500 somatic cotyledons (derived from primary somatic embryos) cocultivated 34 survived two rounds of Kanamycin (100 mg/l) selection
Plate 7. Construction of Rap-RNAi construct of IC84 in pCAMBIA 1300.2 binary vector

7A. Plate of Rap-RNAi cassette of IC84 cloned in pCAMBIA 1382 by SalI and HindIII digestion
Lane 1. Marker. 3.0, 2.0, 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025, 0.001, 0.0005, 0.00025 kb
Lane 2. 3.0 kb (marker)
Lane 3. IC84 (3.0 kb) clone
Lane 4. pCAMBIA 1300.2 (2.0 kb)
Lane 5. plasmid pCAMBIA 1382 (3.0 kb)

7B. Plate of Rap-RNAi cassette of IC84 cloned in pCAMBIA 1382 by SalI and HindIII digestion
Lane 1. Marker. 11.0, 9.9, 9.8, 9.7, 9.6, 9.5, 9.4, 9.3, 9.2, 9.1, 9.0, 8.9, 8.8, 8.7, 8.6, 8.5, 8.4, 8.3, 8.2, 8.1, 8.0, 7.9, 7.8, 7.7, 7.6, 7.5, 7.4, 7.3, 7.2, 7.1, 7.0, 6.9, 6.8, 6.7, 6.6, 6.5, 6.4, 6.3, 6.2, 6.1, 6.0, 5.9, 5.8, 5.7, 5.6, 5.5, 5.4, 5.3, 5.2, 5.1, 5.0, 4.9, 4.8, 4.7, 4.6, 4.5, 4.4, 4.3, 4.2, 4.1, 4.0, 3.9, 3.8, 3.7, 3.6, 3.5, 3.4, 3.3, 3.2, 3.1, 3.0, 2.9, 2.8, 2.7, 2.6, 2.5, 2.4, 2.3, 2.2, 2.1, 2.0, 1.9, 1.8, 1.7, 1.6, 1.5, 1.4, 1.3, 1.2, 1.1, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025, 0.001, 0.0005, 0.00025 kb
Lane 2. 11.0 kb (marker)
Lane 3. IC84 (3.0 kb) clone
Lane 4. pCAMBIA 1300.2 (2.0 kb)
Lane 5. plasmid pCAMBIA 1382 (3.0 kb)
Figure 6A & 6B. Physical map of RNAi vectors, pICR1 and pSCR1
Plate 8. *Agrobacterium* mediated transformation of Cassava immature leaf lobes

A. Explants in preculture medium

B. Co-cultivated leaves in SEIM

C. Initiation of callus in selection medium after 20 days

D. Development of callus after 30 days

E. Separation of embryos from selection medium after 45 days

F. Embryo maturation after 65 days

G. Regeneration initiation after 80 days

H. Regeneration initiation after 100 days

I. Leaf formation after 4 months

J. Shoot elongation after 5 months

K. 8 month old in vitro plants

L. Hardened plants
Plate 9. *Agrobacterium* mediated transformation of somatic cotyledons with SLCMV-Rep-RNAi construct

A. Co-cultivated somatic cotyledons on SEIM
B. Somatic embryo formation after 20 days in selection
C. Embryo germination after 30 days in selection
D. Regeneration after 60 days
E. Leaf formation after 2 months
F. Transgenic plants after 4 months
Plate 10. PCR analysis of putative transgenic lines of Cassava transformed with pSCR2 construct

10A. Amplification of 540 bp 3LCMV:Rep gene from putative transgenic lines
M - Marker, P - Positive control, Lane 1 to 7 - Transgenic plants with pSCR2 construct, Lane 8 - Untransformed cassava

10B. Amplification of 540 bp 3ub plant selectable marker gene from putative transgenic lines
M - Marker, P - Positive control, Lane 1 to 6 - Transgenic plants with pSCR2 construct, Lane 7 - Untransformed cassava
Plate 11. PCR analysis of putative transgenic lines of Cassava transformed with pICR1 construct

11A. Amplification of 620 bp nptII selectable marker gene from putative transgenic lines
M-1kb ladder. P-Positive. Lane 1,2,5 & 6 - Transgenic plants with pICR1 construct. Lane 3 & 4 - Untransformed cassava plants

11B. Amplification of 540 bp Agene from putative transgenic lines
M-1kb ladder. P-Positive. Lane 1,2,5 & 7 - Transgenic plants with pICR3 construct. Lane 3 - Untransformed cassava plant. Lane 4 - Water control.
(Plate 9). The resistant embryos when transferred to somatic embryo maturation medium resulted in germination of 18 embryos (Plate 9C). The germinated embryos when transferred to regeneration medium resulted in regeneration of 6 shoots (Plate 9F) giving a transformation efficiency of 1.2 % (Table 15). Out of 3000 immature leaf lobes cocultivated, 28 calli survived two rounds of Kanamycin (100 mg/l) selection. The resistant embryos when transferred to somatic embryo maturation medium resulted in germination of 19 embryos and further to regeneration medium resulted in regeneration of 9 lines to give a transformation efficiency of 0.3 % (Table 15). But none of the transgenic shoots regenerated were able to survive upto hardening stage.

4.4.3. Molecular analysis of putative transgenic cassava plants of ICMV and SLCMV

4.4.3.1. PCR analysis of SLCMV transgenic plants

The PCR analysis with \textit{hph} gene specific primers amplified an expected size of 620 bp in all the 8 analyzed putative transgenic lines of cassava variety H-226 (Plate 10A). There was no amplification in the non transgenic control. The PCR analysis of transgenic lines with SLCMV \textit{Rep} gene specific reverse primer and \textit{Ocs} terminator reverse primer amplified an expected size of 540 bp in all the 8 analyzed transgenic lines (Plate 10B). There was no amplification in the non transgenic control.

4.4.3.2. PCR analysis of ICMV transgenic plants.

The PCR analysis with \textit{nptII} gene specific primers amplified an expected size of 440 bp in all the 8 analyzed putative transgenic lines of cassava variety H-226 (Plate 11A). There was no amplification in the non transgenic control. The PCR analysis of transgenic lines with ICMV \textit{Rep} gene specific reverse primer and \textit{Ocs} terminator reverse primer amplified an expected size of 540 bp in all the 8 transgenic line (Plate 11B). There was no amplification in the non transgenic control.
Table 14. *Agrobacterium* mediated genetic transformation of cassava for SLCMV resistance

<table>
<thead>
<tr>
<th>Explant</th>
<th>Construct used</th>
<th>No of leaflobes cocultivated</th>
<th>No of embryos survived in 1st selection</th>
<th>No of embryos survived in 2nd selection</th>
<th>No of embryos germinated</th>
<th>No of embryos regenerated</th>
<th>No of lines hardened</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatic cotyledons</td>
<td>pSCR2</td>
<td>500</td>
<td>85</td>
<td>24</td>
<td>14</td>
<td>8</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td>Immature leaf lobes</td>
<td></td>
<td>3000</td>
<td>133</td>
<td>106</td>
<td>64</td>
<td>40</td>
<td>2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

In case of somatic cotyledon explants the data is a mean of 10 experiments with 50 explants in each experiment.

Table 15. *Agrobacterium* mediated genetic transformation of cassava for ICMV resistance

<table>
<thead>
<tr>
<th>Explant</th>
<th>Construct used</th>
<th>No of leaflobes cocultivated</th>
<th>No of embryos survived in 1st selection</th>
<th>No of embryos survived in 2nd selection</th>
<th>No of embryos germinated</th>
<th>No of embryos regenerated</th>
<th>No of lines hardened</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatic cotyledon</td>
<td>pICR1</td>
<td>500</td>
<td>76</td>
<td>34</td>
<td>18</td>
<td>6</td>
<td>-</td>
<td>1.2</td>
</tr>
<tr>
<td>Immature leaf lobes</td>
<td></td>
<td>3000</td>
<td>57</td>
<td>28</td>
<td>19</td>
<td>9</td>
<td>-</td>
<td>0.3</td>
</tr>
</tbody>
</table>

In case of immature leaf lobe explants the data is a mean of 10 experiments with 300 explants in each experiment.
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 (Stanley and gay, 1983). DNA-A has six genes: AC1 encodes a replication-associated protein (Rep) essential for viral DNA replication in association with host DNA polymerase (Hanley-Bowdoin *et al*., 2000); AC2 encodes a transcription activator protein (TrAP) (Sunter and Bisaro, 1992); which also found to have PTGS suppression role in ICMV (Chellappan *et al*., 2004); AC3 encodes a replication enhancer protein (REn) (Sunter *et al*., 1990); AC4 function as PTGS suppressor protein (Chellapan *et al*., 2004). AV1 and AV2 encode coat protein and pre-coat protein, respectively (Padidam *et al*., 1996); and DNA-B has BV1 and BC1 genes that encode a nuclear-shuttle protein (NSP) and movement protein (MP), respectively (Sanderfoot. and Lazarowitz, 1995).

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 (Fregene *et al*., 1997; Mba *et al*., 2001) that have already allowed the identification of a variety of QTLs and a major gene (CMD2) for CMD resistance (Jorge *et al*., 2000, 2001; Akano *et al*., 2002; Okogbenin and Fregene, 2002). 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 and it was further substantiated by Sanford and Johnston in 1985, where they suggested that the transgenic expression of pathogen sequences might interfere with the pathogen itself. 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 (Pooggin et al., 2003).

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 (Chellappan et al., 2004). At ETH the approaches for the control of geminivirus include the expression of antisense viral sequences which interrupt the virus proliferation at the level of DNA replication (Zhang et al., 2003; Vanderschuren et al., 2009). To overcome CMV disease problem, genetic engineering approach provides scope for imparting CMD resistance in Cassava. As a pre-requisite to genetic transformation, high efficiency regeneration protocol was first standardized in Cassava. Then Agrobacterium-mediated transformation method was used to introduce the developed gene silencing constructs into Cassava.

5.1. Cassava regeneration

Somatic embryogenesis is commonly used methods to obtain regenerated plants in cassava. Various explants sources are reported for somatic embryo induction on different media in cassava. Explants like leaf lobes, meristem tips, florets, seeds, cotyledons, zygotic embryo are reported for somatic embryogenesis and regeneration by several research groups in cassava (Stamp & Henshaw, 1987; Raemakers et al., 1993; Li et al., 1995; Mukherjee, 1995, Zhang et al., 2000, Taylor et al., 1996, Raemakers 1993, Groll et al., 2001; Saelim et al., 2006). Seeds and zygotic embryo explants show high
genetic diversity and its sexual propagation results in high genetic segregation in cassava. Hence, the vegetative parts of the cassava plants like immature leaf lobes (for primary somatic embryogenesis) and somatic cotyledons (for secondary somatic embryogenesis) as the most preferred explants for regeneration (Szabados et al., 1987; Raemakers et al., 1993). The present study used two explants such as immature leaf lobes and primary somatic cotyledons in order to standardize the regeneration protocol in cassava.

The explants, immature leaf lobes and somatic cotyledons showed variable responses to somatic embryogenesis and it is mainly influenced by the concentrations of auxins used in the somatic embryo induction medium. Immature leaf lobes showed highest somatic embryogenic induction at 4 mg/l 2, 4-D (54.25%) and 12 mg/l Picloram (58.00%) concentration in independent experiments, whereas, use of other concentration of auxins resulted in less than 30% somatic embryo induction frequency. The frequency of somatic embryo induction increased from 58% to 66.67% in 12 mg/l Picloram containing medium when the MS vitamin was replaced with B5 vitamins. Maximum somatic embryo induction from immature leaf lobes was recorded in MS basal salts with B5 vitamins, 12 mg/l Picloram, 3% Maltose and 4% Phytagel. Many successful reports on somatic embryogenesis in cassava using immature leaf lobes have used Picloram (6 to 12 mg/l), Maltose (3%) and phytagel (0.4-0.6%) for efficient somatic embryogenesis (schopke et al., 1987; Konan et al., 1994; Taylor et al., 1996; Saelim et al., 2006).

In addition to leaf lobes, the older leaves (4th and 5th leaf lobes) were also tested for somatic embryo induction in cassava. Even though the calli growth occurred in the older leaves, however, it showed very few somatic embryos with abnormal development after repeated sub culturing. Similar finding in cassava was reported by Szabados et al (1987). They reported that the importance of the juvenility of the explant tissue in the induction of somatic embryogenesis in cassava. This can be explained by the fact that as the cassava leaves matured, the morphogenic competence also declined, a correlation observed in other regeneration system also (Hakman et al., 1985; Dale, 1980). The present study showed that 1-3 leaf lobes from shoot apical meristem were the most ideal explants for somatic embryo induction.

With a view to produce large number of somatic embryo for cassava regeneration and transformation, secondary somatic embryo induction was attempted. The transfer of green somatic cotyledons developed from primary somatic embryos into fresh somatic embryo induction medium resulted in high frequency secondary somatic embryogenesis induction in cassava. Primary somatic embryogenesis is the process in which embryos are formed from plant explants and secondary somatic embryogenesis is the process in which embryos are formed from embryos (Williams and Maheswaran, 1986). This type of
cyclic embryogenic system will provide frequent and adequate amount of explant material for genetic transformation. The present study revealed that Somatic embryo induction medium with Picloram at 12 mg/l concentration gave maximum production of secondary embryos (80%) from green somatic cotyledons. It is observed that unlike the primary somatic embryos where the somatic embryos are indirectly produced on callus surface, these secondary embryos are directly produced on cotyledon surface without having an intervening callus phase. There are few successful reports of cassava secondary embryogenesis in different cultivars of cassava using somatic cotyledons with Picloram as growth hormone in the somatic embryo induction medium (Raemakers et al., 1993; 1995; Li et al., 1995).

Different stage of the embryos tested for secondary somatic embryogenesis showed that matured green cotyledonal stage embryos as the most suitable starting material. However, the regenerated cotyledonal stage embryo (obtained after transfer to somatic embryo maturation medium) was unsuccessful in secondary somatic embryo induction. This result indicates that the embryogenic competence of embryos which had passed the mature germinated stage was highly reduced. Similar findings are reported in other African cassava cultivars (Raemakers et al., 1993; 1995; Li et al., 1995).

The somatic embryos (both primary and secondary embryos) obtained in 12 mg/l Picloram containing medium were further used for embryo maturation study. Transfer of cotyledonary stage somatic embryo into maturation medium resulted in germination of embryos with cotyledonary embryonic leaves. Among the different somatic embryo maturation media tested, the medium with 0.1 mg/l BAP and 0.02 mg/l NAA resulted in maximum of 80% embryo maturation in two week of incubation. When 1.0 mg/l BAP or higher concentration of BAP was used in maturation medium, the embryos matured within one week, however, the embryos showed abnormal development. It was also noticed that even low concentration of NAA (0.04 mg/l or more) used in maturation medium promoted abnormal root development from the embryos.

The 3-4 week old mature embryo maintained in 0.1 mg/l BAP and 0.02 mg/l NAA failed to grow further in the same medium. Even though large number of very small shoot clusters initiated, it failed to grow further. In order to promote regeneration of mature somatic embryo into complete plant, GA3 was added along with BAP and NAA after 3 weeks of embryo maturation. Among the five different concentrations (0.05 to 0.3 mg/l) of GA3 tested, 0.2 mg/l showed maximum regeneration frequency. Apart from shoot regeneration, the addition of gibberellins also helped for the formation of easily separable shoot
and root structures (bipolar units) from the cassava somatic embryos. Only a few of the embryos in an embryo cluster turned into plantlets, this may be because of the asynchronous development of the embryos and competition between the individual embryos for space and nutrients. The influence of GA3 on shoot elongation was reported in several crop species such as Asparagus cooperi, A. officinalis, Hordeum vulgare and Zea mays (Ghosh and Sen, 1991; Kunitake and Mii, 1990; Kott and Kasha, 1984; Radojevic, 1985). In the absence of gibberellins, the cassava somatic embryos developed a fused cotyledonous structure which was difficult to separate and failed to develop as normal plants. Similar results were discovered in other tuber crops like sweet potato (Cantliffe and Chee, 1990).

5.2. Characterization of CMV causing the CMD in Cassava

In India, cassava is reported to be infected with two important viruses, ICMV and SLCMV (Saunders et al., 2002). Hence, an attempt was made to amplify the Rep gene of ICMV and SLCMV from cassava infected samples collected from Tamil Nadu. PCR analysis confirmed the presence of either one or both the virus from different samples from Tamil Nadu. The PCR amplified product was cloned and the DNA sequencing confirmed the presence of ICMV and SLCMV infection in cassava. The SLCMV Rep gene showed 95 to 100% sequence identity to the already deposited SLCMV sequence in the NCBI Genbank. In case of ICMV, the cloned Rep gene sequence showed 92 to 100% sequence identity to the already deposited sequence in the NCBI database. Also the distribution of ICMV and SLCMV varies from one district to other. The result shows that samples from Salem district of Tamil Nadu is predominantly infected with SLCMV and few samples showed ICMV infection. In addition to individual virus infection from cassava samples of Salem district, some of the samples showed the mixed infection of both ICMV and SLCMV. This is in contrast to earlier report by Patil et al., 2005, that Indian cassava mosaic virus was more predominant at Salem.

In case of samples from Coimbatore district of Tamil Nadu showed the presence of ICMV in most of the samples analyzed, with SLCMV infection and mixed infection in few samples. This is in contrast to earlier report by Patil et al., 2005, that SLCMV was more predominant at Coimbatore district. Mixed infection of ICMV and SLCMV was observed in approximately 20% of the samples collected from all the five district of Tamil Nadu. Earlier it was reported that ICMV was restricted to the northern and central districts of Kerala, whereas SLCMV was more widespread in all the CMD-affected regions surveyed, even in the few isolated CMD-affected pockets of Andhra Pradesh (Patil et al., 2005). The present study shows
that both ICMV and SLCMV were present throughout the major cassava growing areas in Tamil Nadu. Earlier reports showed that SLCMV is mainly responsible for cassava mosaic disease in Tamil Nadu (Patil et al., 2005). However, the present study revealed the widespread nature of ICMV in an area which is originally colonized by SLCMV. In addition to this, a recent report shows that a new strain of ICMV is discovered which can infect Jatropha (*Jatropha curcas*) apart from cassava (Gao et al., 2010). Such a scenario can give rise to recombination or synergism between the existing viruses, resulting in a situation similar to the African CMD epidemic (Pita et al., 2001; Thresh et al., 1998; Zhou et al., 1997).

Compared to SLCMV, ICMV showed much divergence pattern when their Rep gene sequences are analyzed for homology. The isolates Erode, Coimbatore, Salem and Kerala have a high identity among individual cluster (99-100%) but each group showed less identity when compared group wise. Such pattern of nucleotide identity was not observed in SLCMV isolates and they tend to show an identity of 98-100%.

Many reports have demonstrated that RNAi can be engineered to target viral RNA in plants (Smith et al., 2000; Tenllado and. diaz-ruiz, 2001). It is reported that PTGS is highly safe to host plant and it is not associated with negative effect on plant growth and development sometimes observed in case of viral protein expression. As a proof of the concept that RNAi can be engineered to effectively target DNA virus demonstrated in Mung Bean Yellow Mosaic Virus (MYMV-Vig) (Pooggin et al., 2003). Similarly, a PTGS-based strategy to control DNA virus replication was demonstrated when plant cells simultaneously transfected with African Cassava Mosaic Virus (ACMV) and with a synthetic siRNA designed to target the AC1 gene. There was over a 90% reduction in the levels of AC1 mRNA and 70% reduction in viral DNA as compared with controls (Vanitharani et al., 2003). It is now well established that both RNA and DNA viruses can be controlled by RNAi approach. Unlike the RNA virus, where only PTGS is mainly used for virus control, the DNA virus control involves both by TGS and PTGS (Vanitharanai et al., 2005). Reports on siRNA accumulation in tomato plants infected with the monopartite Geminiviruses TYLCV (Lucioli et al., 2003; Ramesh et al., 2007) and in cassava plants infected with the bipartite geminivirus ACMV (Chellappan et al., 2004) clearly reflect the role of the RNAi pathway as a natural defense mechanism against these DNA viruses.

It is well documented by several reports that RNAi technology is superior to antisense, sense or protein mediated resistance. In RNA interference, initially a double stranded RNA (dsRNA) is recognized as
a substrate for the subsequent targeting and degradation of sequence-homologous RNAs. A natural recovery from ACMV-[CM] infection and its correlation with high levels of virus-derived siRNA accumulation support PTGS as a strategy to control Geminiviruses effectively (Chellappan et al., 2004).

Hence, in the present study, RNAi approach which targets the Rep gene of ICMV and SLCMV was followed to engineer resistance in cassava variety H-226. Rep gene is very essential for the Geminivirus viral DNA replication. The geminivirus 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 (Saunders et al., 2002; Stenger et al., 1991). 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 (Chellappan et al., 2005). 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 (Aragao et al., 1998; Asad et al., 2003; Yang et al., 2004). Furthermore, it has been already reported that the transient expression of full length AC1 gene of ACMV (Chellappan et al., 2004) 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 (Hong et al., 1996; Sangere et al., 1998; Ramesh et al., 2007).

Since the Rep gene of ICMV and SLCMV showed high sequence variability, control of ICMV and SLCMV using RNAi was attempted using separate RNAi vectors. To achieve the RNAi mediated resistance in cassava, a 440 bp region of ICMV and SLCMV Rep gene which overlap with the AC4 gene was used for vector construction. Wesley et al. (2001) 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 (Smith et al., 2000; Wesley et al., 2001). 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 pCR1 and pSCR2) targeting the Rep gene of ICMV and SLCMV respectively were transformed into Cassava variety H-226.

5.3. *Agrobacterium*-mediated transformation of cassava variety H-2226

*Agrobacterium*-mediated transformations of cassava with developed RNAi constructs were done using the immature leaf lobes and green somatic cotyledon explants. ICMV specific RNAi-Rep gene cassette was cloned into two binary vectors, one containing the kanamycin resistant gene (*nptII*) and other containing the hygromycin resistant gene (*hph*). Both the vectors were used for co-cultivation. Compared to immature leaf lobes the somatic cotyledons were able to survive the Agrobacterium infection. In case of Immature leaf lobes, 50% of the explant turned yellow and dried after Agrobacterium infection, whereas, more than 90% of the cotyledonous explants showed normal growth after infection. Hence the transgenic recovery was more in somatic cotyledonous explants (2.8%) compared to the immature leaf lobes where 1.5% transformation efficiency was obtained. Earlier reports also indicate somatic cotyledon explants or embryogenic cell suspension as ideal starting material for genetic transformation of cassava (Li et al., 1996; González et al., 1998, Zhang et al., 2000, 2005). Even though it was found that both hygromycin and kanamycin can be used in the described protocol, the use of hygromycin was more preferred for transgenic cassava selection since low concentration of hygromycin gave good selection of the transformed embryos.

The use of somatic cotyledons also reduced the time required to recover the putative transgenic plants of cassava. By using somatic cotyledons the transgenic plants can be obtained in 9 months whereas from immature leaf lobes the transgenic plants are obtained in 11 months. Also the transformation protocol using the immature leaf lobes were very laborious where continuous production of leaf lobes should be required using healthy *in vitro* plants. In the case of somatic cotyledons the explants are relatively easy to develop and maintain through the cyclic somatic embryogenic system established in cassava variety H-226.

The transgenic plants integrated with the ICMV and SLCMV Rep- RNAi constructs were analyzed using PCR amplification of the selectable marker genes (hygromycin and kanamycin primers) and Rep gene specific primers. The Rep gene was amplified by using the Rep reverse and *ocs* terminator reverse primers. As the *ocs* gene specific primer will not amplify the Rep gene of infected plants, hence it may be
considered that the amplification from these primer combinations is due to true integration of transgene in the transgenic plants. The putative transgenic plants transformed with SLCMV specific gene silencing construct are showing normal growth and development at *in vitro* conditions. Further assays on the transgenic plants are under progress.
CHAPTER VI
SUMMARY

1. Regeneration protocol standardized for cassava variety, H-226 using immature leaf lobes as explant
2. A cyclic somatic embryogenic system is established in cassava variety, H-226 using somatic cotyledon as explant
3. PCR analysis confirmed the presence of ICMV and SLCMV infection in samples collected from five major cassava growing districts of Tamil Nadu. The analysis also showed that approximately 20% of the samples from five districts showed mixed infection of ICMV and SLCMV.
4. A total of 11 isolates of ICMV and 15 isolates of SLCMV Rep gene were cloned and confirmed by DNA sequencing. The isolated ICMV Rep gene sequences showed a nucleotide identity which varied from 92 to 100% with already reported ICMV Rep gene sequences available in the NCBI database. The isolated SLCMV Rep gene sequences showed a nucleotide identity which varied from 95 to 100% with already reported SLCMV Rep gene sequences available in the NCBI database. These sequences of ICMV and SLMCV Rep gene are submitted to NCBI database.
5. In total four RNAi gene constructs were made for the control of CMV. Two vectors targeting the Rep gene of ICMV and two vectors targeting the Rep gene of SLCMV were made for genetic transformation of cassava.
6. Agrobacterium mediated transformation of cassava, variety H-226 done using the developed RNAi construct targeting the 5’ end of the ICMV and SLMCV Rep gene.
7. In total, 48 putative transgenic plants were generated to obtain SLCMV resistance. Out of 48 shoots, 2 plants are hardened in greenhouse. In total, 15 putative transgenic plants were generated to obtain ICMV resistance. None of the transgenic shoots survived up to hardening stage. The presence of the transgene was confirmed in some of the selected transformed plants by PCR analysis.
REFERENCES


Lucioli, A. et al. (2003) Tomato yellow leaf curl Sardinia virus derived resistance to homologous and heterologous geminiviruses occurs by different mechanisms and is overcome if virumediatedtransgene silencing is activated. *J. Virol.** 77, 6785–6798


## Appendix 1

### Media and stock composition of MS media used

<table>
<thead>
<tr>
<th>Compounds</th>
<th>mg/l of media</th>
<th>Stock (g/l)</th>
<th>Volume of stock used per liter of media</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients (40X)</strong></td>
<td></td>
<td></td>
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<tr>
<td>NH$_4$NO$_3$</td>
<td>1650</td>
<td>66</td>
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</tr>
<tr>
<td>KNO$_3$</td>
<td>1900</td>
<td>76</td>
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<tr>
<td>MgSO$_4$. 7H$_2$O</td>
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</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>170</td>
<td>6.8</td>
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</tr>
<tr>
<td>CaCl$_2$. 2H$_2$O</td>
<td>440</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td><strong>Micronutrients (200X)</strong></td>
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<td></td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>6.2</td>
<td>1.24</td>
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</tr>
<tr>
<td>CoCl$_2$. 6H$_2$O</td>
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<td>0.005</td>
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</tr>
<tr>
<td>CuSO$_4$. 5H$_2$O</td>
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<td>0.005</td>
<td></td>
</tr>
<tr>
<td>MnSO$_4$. 4H$_2$O</td>
<td>22.3</td>
<td>4.46</td>
<td></td>
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<tr>
<td>Na$_2$MoO$_4$.2 H$_2$O</td>
<td>0.25</td>
<td>0.05</td>
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<tr>
<td>ZnSO$_4$. 7H$_2$O</td>
<td>8.6</td>
<td>1.72</td>
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<tr>
<td>KI</td>
<td>0.83</td>
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<tr>
<td>Na$_2$EDTA</td>
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<tr>
<td>FeSO$_4$. 7H$_2$O</td>
<td>41.7</td>
<td>4.17</td>
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<tr>
<td><strong>Organic supplement (200X)</strong></td>
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<tr>
<td>Glycine</td>
<td>2.0</td>
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<tr>
<td>Nicotinic acid</td>
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<tr>
<td>Pyridoxine HCl</td>
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<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
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<td></td>
</tr>
<tr>
<td>Myo-inositol</td>
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</tr>
</tbody>
</table>
Approximately 100 – 200 mg of diseased cassava leaf tissue was placed into a plastic bag and frozen in liquid nitrogen. The tissue was ground immediately using pestle and mortar mixed with 1 - 2 ml of cetyl trimethyl ammonium bromide (CTAB) extraction buffer (Annexure II). About 750 µl of the sample was poured into a 1.5 ml eppendorf tube and kept at 65 °C for 10 min. The samples were then mixed with an equal volume (750 µl) of chloroform: isoamylalcohol (24:1) and centrifuged at 10,000 g for 10 min. The top aqueous phase was transferred into a new 1.5 ml eppendorf tube and equal volume of chloroform: isoamylalcohol (24:1) was added and mixed and centrifuged again at 10,000 g for 10 min. Three hundred µl of aqueous phase was transferred into new eppendorf tube and DNA was precipitated by adding 2 volumes (600 µl) of ice cold (−20 °C) ethanol and 0.5 volumes (150 µl) of 5M NaCl. The mixture was incubated at −20 °C for at least 1 h. The samples were centrifuged at 12,000 g at 4 °C for 10 min and the supernatant was discarded. The pellet was washed in 0.5 ml 70% ethanol by vortexing and then centrifuged for 5 min at 12,000 g. The ethanol was removed and the pellet was dried for 5 min. The dried pellet was suspended in 50 µl of 0.1X TE buffer (or sterile distilled water and stored at −20 °C.
Appendix 3

Physical map of pJET1.2 vector
Appendix 4

Figure physical map of pHANNIBAL vector
Appendix 5

Physical map of pBLUESCRIPT/SK+ vectors
Appendix 6

Physical map of pCAMBIA1305.2 binary vector
Appendix 7

Genomic DNA isolation from Agrobacterium (Chen and Kuo, 1993)

A loopful of Agrobacterium culture was inoculated in YEP broth with kanamycin (100 mg/l) and rifampicin (10 mg/l) and incubated on a rotary shaker (220 rpm) at 28 °C for 15-18 h or till 1.0 O.D600 growth. Then, the culture was centrifuged at 12000 rpm for 2 min. The pellet was vortexed in a minimal amount of supernatant retained and mixed well with 200 µl of lysis buffer (100mM Tris-acetate pH 7.8, 20mM sodium acetate, 1mM EDTA, 1% SDS). To this, 66 µl of 5M NaCl was added, mixed well and kept at -20 °C for 10 min. The mixture was centrifuged at 12000 rpm for 10 min at room temp. To the supernatant, equal volume of phenol : chloroform (1:1) was added and mixed well till a milky emulsion appeared. It was centrifuged at 12000 rpm for 10 min at room temp. To the aqueous phase equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged. To the top layer, 2.5 volume 99.9% (v/v) ethyl alcohol was added and left at -20 °C for overnight or -70 °C for 1 h. Then, the tubes were centrifuged at 12000 rpm for 10 min. After a washing with 70% ethanol, pellet was air dried for 30 min, dissolved in 40 µl of 0.1X TE (1mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0) and treated with 1-2 µl of RNase A (10 mg/ml stock) for further use.