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Development of putative transgenic lines of cassava variety H-226

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Cassava production in India is drastically affected by cassava mosaic disease (CMD) caused by the Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV). An attempt was done to develop transgenic cassava lines resistant to SLCMV through RNAi vector targeting a conserved 440 bp of 5' end of SLCMV *Rep* (AC1) gene which also overlaps with part of AC4 gene, and functions as a viral RNAi suppressor protein. The partial *Rep* gene of SLCMV was cloned in sense and anti-sense orientations in the RNAi intermediate vector, pHANNIBAL and finally mobilised into binary vector pART27, to construct pSCR1 which contains the kanamycin-resistance gene as a plant selectable marker. In order to use hygromycin as selection agent in cassava genetic transformation, Rep-RNAi gene cassettes of SLCMV was cloned into pCAMBIA1305.2 and the constructs was named pSCR2. *Agrobacterium* mediated cocultivation of cassava embryogenic calli was done with the developed RNAi constructs using two different explants namely, immature leaf lobes and somatic cotyledons. In total, 48 putative transgenic cassava shoots were regenerated on a regeneration medium containing 30 mgl/l hygromycin, of which 2 putative transgenic plants were transferred for hardening. All the putative transgenic cassava plants were PCR-positive for *hph* gene and *Rep* gene indicating integration of transgenes of interest.

Key words: RNAi constructs, cocultivation, cassava mosaic disease (CMD).

INTRODUCTION

Cassava (*Manihot esculenta*) is a major tuber crop cultivated in 13 states of India. Cassava is grown in an area of 2.34 mha in India, with Kerala ranking first in area (1.04 mha) followed by Tamil Nadu (0.95 mha) (FAOSTAT, 2010). Cassava production is affected by a combination of biotic and abiotic stresses, among them; cassava mosaic disease (CMD) caused by cassava mosaic geminivirus (CMG) limit the productivity of cassava (Pita and Fauquet, 2001). CMD had not been reported in India before 1966 and has become more prevalent in recent years in Southern India especially Salem, Dharmapuri districts of Tamilnadu and almost all

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Abbreviations: SEIM, Somatic embryo induction medium.

Table 1. Primers designed for amplification of sense and Antisense of Rep gene of SLCMV used for RNAi vector construction.

S/N	Particulars	Primer (SLCMV)	Primer Position
1	SLCMV Sense FP	5'CCC***CTCGAG*** CCCACAAACCCAAAATTCA 3'	112 –131
2	SLCMV Sense RP	5'CCC***GGTACC*** ATACGGAGGTGGTGGTGTT 3'	521- 540
3	SLCMV Antisense FP	5'CGC***GGATCC*** CCCACAAACCCAAAATTCA 3'	112 –131
4	SLCMV Antisense RP	5'CCC***AAGCTT*** ATACGGAGGTGGTGGTGTT 3'	521- 540

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 (such as H-226, SreeHarsha) or tolerant (for example, H165, Co-1, Co-2 and MDV2) to the disease. None of the varieties grown in Tamil Nadu are resistant to the disease. Conventional cassava breeding is not successful in developing resistant varieties, which is seriously limited due to long growth cycle, highly heterozygous nature, poor seed set and viability.

Cassava Mosaic Disease (CMD) in India is caused by two species of virus namely, Indian Cassava Mosaic Virus (ICMV) and Sri Lankan Cassava Mosaic Virus (SLCMV) (Patil et al., 2005). Both the DNA viruses belonging to the bipartite begomovirus genus of the family *Geminiviridae*, and are transmitted by whitefly *Bemisia tabaci* Genn. 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., 2001; Calvert and Thresh, 2002). 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.

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 (References). 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 (Patil et al., 2011; Yadav et al., 2011), whereas, the DNA viruses are effectively controlled by silencing the *Rep* gene, which is indispensable for DNA replication of virus (Pooggin et al., 2003). 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 *Rep* gene), which is indispensable for the replication of virus and disease development. This is the first attempt to develop transgenic cassava resistant to CMG using RNAi approach in India.

MATERIALS AND METHODS

Construction of gene silencing vectors with *npt* and *hph* selectable marker genes

The hairpin binary vectors are made using CSIRO RNAi vector construction steps using pKANNIBAL vector. A conserved sequence of size 440 bp from the 5' region of the Rep gene sequence of SLCMV were identified and primers were designed to amplify 440 bp Rep gene of ICMV covering 112 – 540 nt region of Rep gene (Table 1). The restriction enzymes, *Xho*I and *Kpn*I were appended with sense forward and reverse primer and *BamH*I and *Hind*III were appended with antisense forward and reverse primer of SLCMV respectively. The PCR product is then cloned sequentially on either side of the pdk intron to become the two arms of the hairpin. The complete hairpin RNAi gene cassette was released by digestion with *Not*I restriction enzyme and cloned into pART27, plant transformation binary vector from CSIRO Plant Industry, Australia. The constructed pART27 vectors with RNAi-SLCMV Rep cassette was designated as pSCR1. Also, the RNAi cassette of SLCMV was further cloned into the *Not*I site of pBLUESCRIPT (SK+) vector and then the insert was released with *Sal*I and *Sac*I restriction digestion and cloned into pCAMBIA1305.2 which has *hph* selectable marker gene, hygromycin B (Hy) phosphotransferase-encoding gene. The recombinant clones were identified by restriction analysis with *Sal*I and *Sac*I enzyme. The recombinant plasmids were named pSCR2 (Figure 1).

Bacterial strains and culture conditions

E. coli conjugative helper strain, DH5α harbouring pRK2013 were grown separately on LB agar plates containing Spectinomycin (100 mg/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 10 mg/l. The RNAi-Rep vector was mobilized into Agrobacterium strain LAB4404 using triparental method.

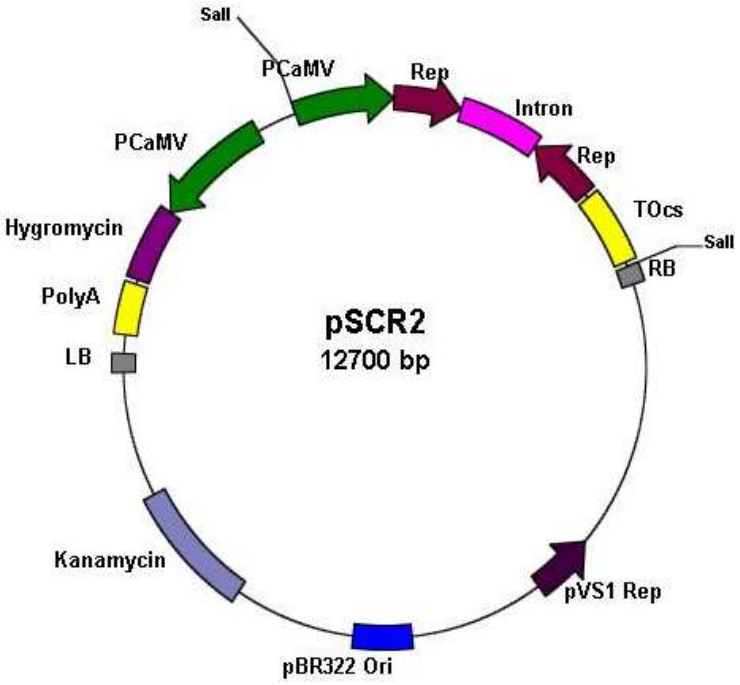

Figure 1. Physical map of RNAi vector pSCR2.

Cocultivation

Young immature leaf lobes of 0.5 to 1 cm in size just near to the apical meristem of cassava 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 SEIM) were used for cocultivation. These explants were precultured on SEIM for 2 days in dark at 28°CA donor *E. coli* strain DH5α harbouring the recombinant plasmid, pSCR2 The *Agrobacterium* strain *LBA4404* harbouring 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 blot dried on filter paper and transferred to callus induction medium containing MS salts, B5 vitamins, 30 g/l maltose, 12 mg/l Picloram, 300 mg/l Cefotaxime and 30 mg/l Hygromycin as selection agent.

Selection and plant regeneration

After 48 h of cocultivation, the treated explants were kept in SEIM with hygromycin (30 mg/l) and cefatoxime (300 mg/l). The cultures were incubated in the dark at 26°C. Two rounds of selection were done to obtain transformed tissue. The sub culturing was done at 20 days interval. The somatic embryos developed at the end of second subculture in SEIM were transferred to somatic embryo maturation medium. 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 GA_3). 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.

Molecular analysis of putative transgenic lines of cassava

Crude DNA was extracted from small leaf bits (2-3 cm) which 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. after centrifugation at 12,000 rpm for 10 min, pellets were air dried at room temperature and dissolved in 30 µl of 0.1X TE buffer (1.0 mMTris-HCl pH 8.0 and 0.1 mM EDTA pH 8.0; Proebski et al., 1997). 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 mMTris-HCl pH 8.8, 50 mMKCl and 1.5 mM $MgCl_2$), 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 reactions are carried out in 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

Table 2. *Agrobacterium* mediated *genetic* transformation of cassava for SLCMV resistance.

Explant	No of explants cocultivated	No of embryos survived in 1st selection	No of embryos survived in 2nd selection	No of embryos germinated	No of embryos regenerated	No of lines hardened	Transformation efficiency (%)
Somatic cotyledons	500	85	24	14	8	-	1.6
Immature leaf lobes	3000	133	106	64	40	2	1.3

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 using the SLCMV Rep gene specific primers with the following temperature conditions, pre-incubation period at 94 °C for 5 min, leading to 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and synthesis at 72°C for 1 min, followed by extension at 72°C for 5 min.

RESULTS

Genetic transformation of cassava variety H-226 for SLCMV resistance

Selection and plant regeneration

Two explants immature leaf lobes and somatic cotyledons were tried for genetic transformation using agrobacterium strain LBA4404 containing the RNAi-SLCMV Rep gene cassette in pSCR2 with *hph* as gene selectable marker. Out of 3000 immature leaf lobes co cultivated, 106 calli survived two rounds of Hygromycin (30 mg/l) selection. The resistant embryos when transferred to somatic embryo maturation medium resulted in germination of 64 embryos and further to regeneration medium resulted in regeneration of 40 lines to give a transformation efficiency of 1.6% (Table 2). 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 2). In total, out of the 48 transgenic shoots developed two shoots developed well and were hardened in greenhouse (Figures 2 and 3).

Molecular analysis of putative transgenic cassava plants by PCR

The transgenic plants integrated with the SLCMV Rep-RNAi constructs were analysed using PCR amplification of the selectable marker genes (hygromycin and kanamycin primers) and Rep gene specific primers. The PCR analysis with *hph* gene specific primers amplified an expected size of 620 bp in all the 8 analyzed putative transgenic lines of cassava variety H-226 (Figure 4B). 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 PCR analysis of transgenic lines with SLCMV *Rep* gene specific reverse primer and *Ocs* terminator reverse primer amplified an expected size of 540 bp in all the 8 analyzed transgenic lines (Figure 4A). 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.

DISCUSSION

Cassava (*Manihot esculenta)* is a staple food for 600 million people in the tropical and subtropical belt, as well as a feedstock for numerous industrial applications, including food, feed and starch. Cassava production in India is seriously hampered by the occurrence of two strains of cassava mosaic virus, ICMV and SLCMV leading to a serious decline of the crop and drastic yield reduction (Reference?). 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, SreeHarsha) or moderately tolerant (includes H-165, Co-1, Co-2 and MVD2) to CMG. The high heterozygosity and inbreeding depression complicates 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

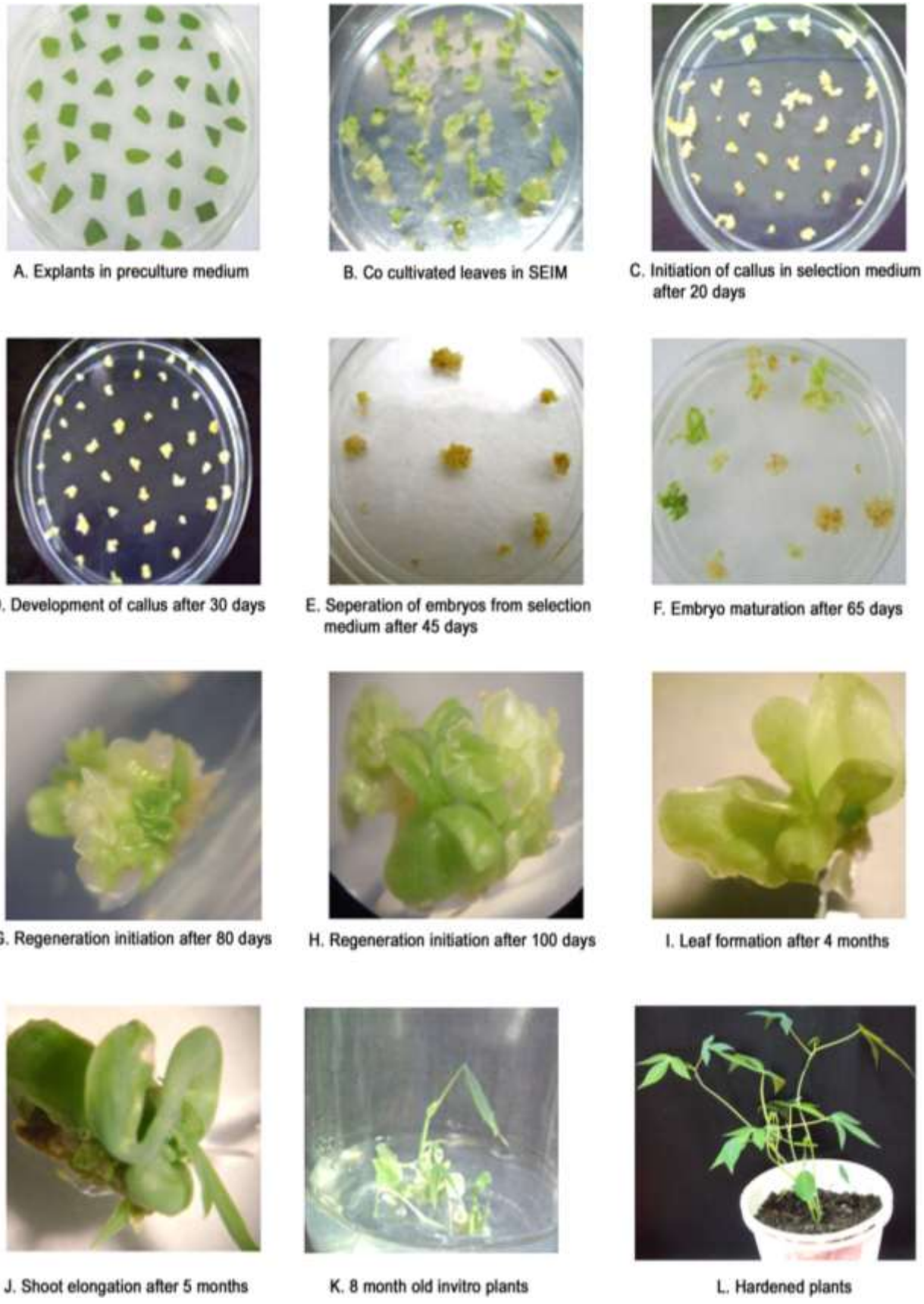

Figure 2. Agrobacterium mediated transformation of cassava immature leaflobes.

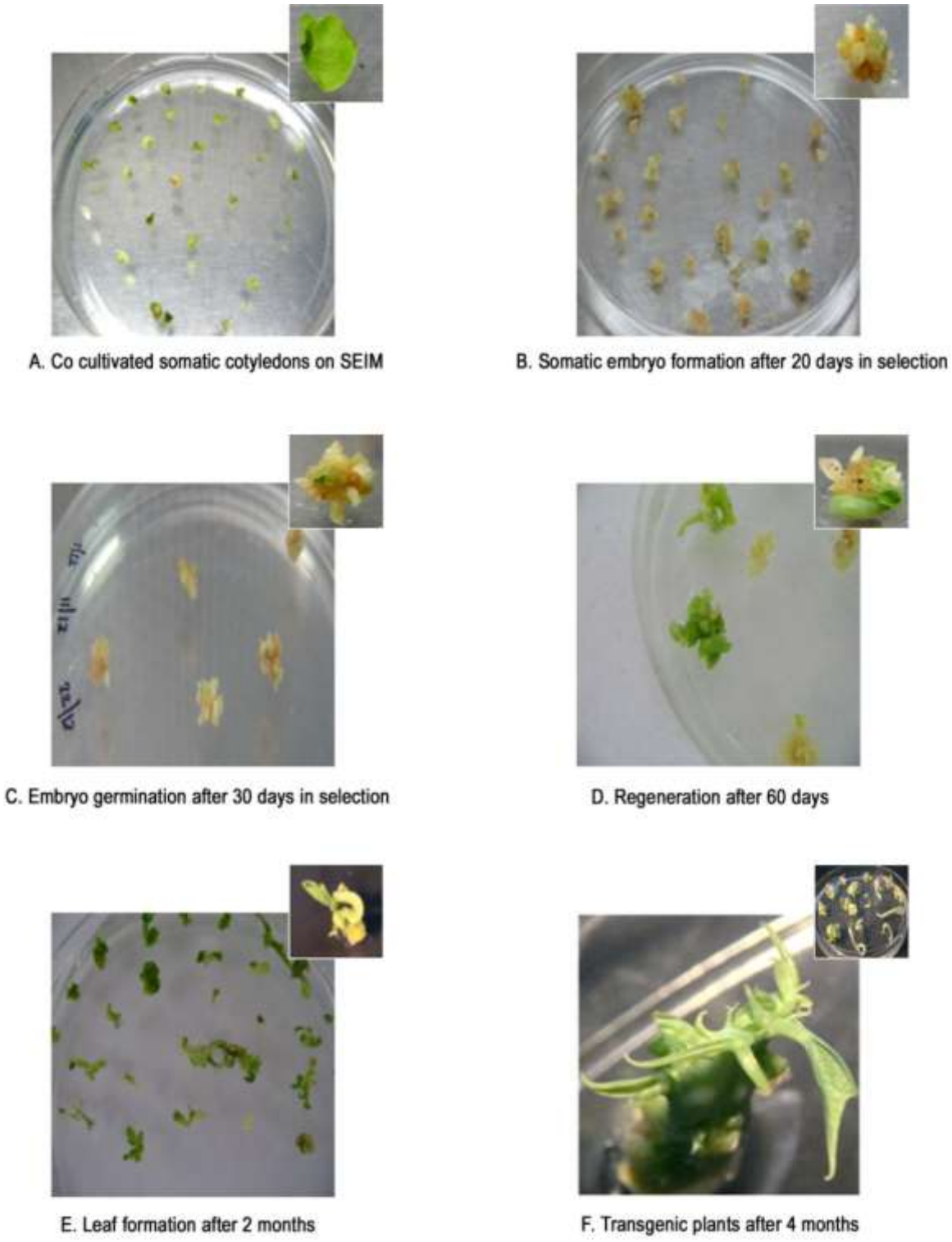

Figure 3. Agrobacterium mediated transformation of somatic cotyledons.

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

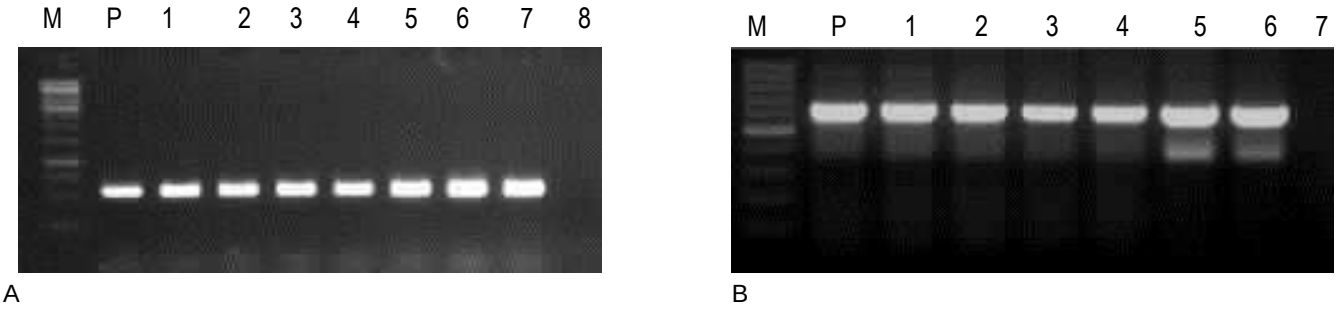

Figure 4. PCR analysis of putative transgenic lines of Cassava transformed with pSCR2 construct. **A.** Amplification of 540 bp SLCMV Rep gene from putative transgenic lines. M, Marker. P, Positive control. Lane 1 to 7, Transgenic plants with pSCR2 construct. Lane 8 –Untransformed cassava. B. Amplification of 640 bp hph 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.

cassava. 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). To overcome CMV disease problem, genetic engineering approach provides scope for imparting CMD resistance in Cassava.

Agrobacterium-mediated transformations of cassava with developed RNAi constructs were done using the immature leaf lobes and green somatic cotyledon explants. SLCMV 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*). Initially one of the vectors was used for cocultivation studies. Even though the explant somatic cotyledons were able to survive the Agrobacterium infection those transgenic plants failed during greenhouse hardening. 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. The transformation efficiency was more in the case of somatic cotyledons but transgenic recovery were poor. The use of immature leaf lobes for transformation is a laborious process hence ways are under progress to develop friable embryogenic calli which can be preserved for long purpose.

Conflict of Interests

The authors have not declared any conflict of interests.

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