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# ISOLATION, CLONING AND AGROBACTERIUM MEDIATED TRANSFORMATION OF MYMV REPLICASE (REP) GENE IN *VIGNA RADIATA* (L.) WILCZEK FOR MYMV RESISTANCE

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**Abstract–**The binary plant cloning vector pCAMBIA1305.2 carrying the MYMV *Replicase (Rep)* gene was constructed and transformed into a competent *Agrobacterium tumefaciens*, strain LBA4404 and was used for co-cultivation of mungbean varieties, KKM-3, IC-39340-1, China mung and LM-1668 for imparting mungbean yellow mosaic virus (MYMV) resistance. The putative transformants ($T_o$ generation) were selected on shooting media (SM) supplemented with 5 $mgl^{-1}$ BAP, 50 $mgl^{-1}$ hygromycin and 250 $mgl^{-1}$ cefotaxime. The multiple shoots were inoculated on shoot elongation media (SEM) with 0.3 $mgl^{-1}$ zeatin and 2.5 $mgl^{-1}$ BAP for elongation and rooted on rooting media (RM), comprising half MS supplemented with 0.2 $mgl^{-1}$ NAA. The GUS assay and genomic PCR analysis of the $T_o$ transformants revealed, four positive putative transgenic lines for *Rep* gene specific primers and had a positive correlation with vector specific *hptII* primers. Likewise, VirG amplification revealed no *Agrobacterium* contamination in the apoplast of all the putative $T_o$ transformants.

## INTRODUCTION

Mung bean or green gram or dal is an important legume crop of Indian food habit and source of protein in daily diet. It is a short duration rainfed crop mostly cultivated throughout Indian sub-continent and also parts of South east and East Asia regions. It belongs to *Vigna* genus and sub-genus Ceratotropis (Lambrides and Godwin, 2007). India is the major mungbean producing country where the cultivation area is around 3.8 Mha with annual production of 2.46 MT of grain (Directorate of Economics and Statistics, India, 2019-20). Besides human food consumption it is being used as animal fodder and in crop rotation as a boon for nitrogen fixation.

The crop productivity of mungbean is affected by several challenges in the form of abiotic and biotic stresses. The impact of biotic stresses like various pest and diseases is enormous and among which the viral diseases like mungbean yellow mosaic virus (MYMV) cause devastation to all the mungbean growing areas (Borah and Dasgupta, 2012).

MYMV is single stranded bipartite DNA virus belonging to the genus *Geminivirus*, family: *Begomoviridae* and transmitted in a circulative persistent manner by insect vector, whitefly (*Bemisia tabaci*), (Ilyas *et al*., 2009). Mungbean yellow mosaic India virus (MYMIV), mungbean yellow mosaic virus (MYMV) and horse gram yellow mosaic virus (HgYMV), are accepted as causal agents of MYMD in different regions of the world (Qazi *et al*., 2007; Malathi and John 2008a; Ilyas *et al*., 2010). The control of the disease can be done by management of virulent insect vectors, which is an indirect and less promising.

Conventional breeding for disease resistance in legumes is limited due to several constraints like, lack of availability of genetic variability (Yadav *et al*., 2012), very little knowledge in genomic research of mungbean compared to other legumes, lack of promising MYMV resistant QTLs and exploitation of the *R genes* from wild varieties leading to negative trait drag in subsequent populations of F2 and RILs. However, development of transgenic MYMV resistant varieties and their incorporation into traditional cultivars is a promising approach (Karthikeyan *et al*., 2014).

The ORFs of MYMV consists of several essential genes like coat protein (CP), replication associated protein (Rep), movement protein (MP), transcription trans-activator protein (TrA) and nuclear shuttle protein (NSP) (Snehi *et al*., 2015), exploiting them for MYMV resistance through PDR (pathogen derived resistance) mediated approach can provide alternative solution to the conventional breeding to impart resistance. With this preview, the present study was undertaken with an aim to incorporate the *Rep* gene into a binary vector by *Agrobacterium* mediated transformation with strain LBA4404 in *V. radiata.*

## MATERIALS AND METHODS

### Genomic DNA isolation and amplification of *Rep* gene

The MYMV infected leaf samples were collected from the field and the genomic DNA was isolated by SDS method (Islam *et al*., 2011; Swapnil and Peter, 2015) and amplified by the MYMV *Rep* gene specific primers which were designed by using Snap Gene software (version 4.1.9) and gene source was from NCBI database (Acc. No.: DQ400848.1), Forward primer: 5'ATGCCTAGACTCGGTCGTTTG3' and Reverse primer: 5'TCAATTCGAGCGTCGAATTGC3' (Sigma-Aldrich, USA), respectively PCR reactions were carried out with a 20 µl reaction mixture containing 10x PCR buffer with 20 mM $MgCl_2$, 2.5 mM dNTPs, deionized nuclease free water, forward and reverse primers, DNA template and 1U/µL Taq polymerase (BlackBio Biotech 3B DNA polymerase, India Ltd.). The PCR amplification of DNA was performed using thermal cycler (Bio-Rad Laboratories, USA), under the given conditions of initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45s, annealing temperature at 59 °C for 1 min, extension at 72 °C for 1 min 45s and final extension reaction at 72 °C for 10 min and hold. The amplified DNA fragments were then analyzed by electrophoresis using agarose gel 1per cent and visualized upon ethidium bromide staining (Sambrook *et al*., 1989) under UV gel documentation unit (Alpha InnonTech, USA) and ladder used was $1Kb^+$ step up ladder (GeNei Bangalore, Catalog No.: 2652070501730)

### Construction of recombinant binary vector with MYMV *Rep* gene

The amplicon of MYMV *Rep* gene was purified by GeneJET PCR purification kit (Thermo Scientific, Inc., USA), cloned into pTZ57RT cloning vector (InsTAclone PCR cloning kit, Thermo Scientific, Inc., USA) and a construct pTZ57RT::MYMV *Rep* was transformed into competent *E. coli* DH5α cells, following standard protocols (Sambrook and Russell, 2001) and incubated at 37 °C for 24 h on LB agar with appropriate selection antibiotic, ampicillin 100 $mgl^{-1}$. The positive recombinant clones were identified by blue-white screening of colonies, confirmed by colony PCR amplification using gene-specific *Rep* primers and by restriction analysis. For PCR confirmation of clones, colonies were selected, diluted in deionized nuclease free water and used as the template. The plasmid DNA from positive colonies were isolated by GSure Plasmid Mini Prep Kit (GCC Biotech India) and used as the template for the PCR amplification. The restriction analysis was done using SacI-HF and HindIII-HF restriction enzymes (NEB, UK). The MYMV *Rep* gene cloned into pTZ57R/T was sequenced using M13 forward and reverse primers, for confirmation the sequence was analyzed using BLAST algorithm (https://www.ncbi.nlm.nih.gov/). The MYMV confirmed *Rep* gene of pTZ57RT clone was sub-cloned into a plant binary vector pCAMBIA1305.2, between the SacI and HindIII restriction site driven by CaMV35s enhanced promoter with $Hyg^r$ selection marker gene and GUS reporter gene between right and left T-DNA borders (Fig. 2a). This construct, pCAMBIA1305.2::CaMV35s::MYMV *Rep*::Nos was transformed into competent *E. coli* DH5α cells. The transformants were confirmed by colony PCR, plasmid PCR amplification and restriction analysis.

### Transformation of *Agrobacterium* with pCAMBIA1305.2::CaMV35s::MYMV *Rep*:: Nos

The confirmed clone containing the recombinant binary vector construct, pCAMBIA1305.2::CaMV35s::MYMV *Rep*::Nos was, transformed into *Agrobacterium tumefaciens* strain LBA4404 by freeze thaw method (Jyothishwaran *et al*., 2007) and incubated at 28°C for 48 h on YEP agar medium with appropriate selection antibiotics, rifamycin 100 $mgl^{-1}$ and 50 $mgl^{-1}$ kanamycin. Transformed *Agrobacterium* were confirmed by colony PCR and plasmid PCR amplification with *Rep* gene specific primers. The positive colony of confirmed recombinant *Agrobacterium* was used for co-cultivation of mungbean explants for *Agrobacterium* mediated transformation.

*Agrobacterium* mediated genetic transformation of

Mungbean varieties

**Seed material:** The seeds of MYMV susceptible mungbean varieties (Swapnil Shahakar, 2018), KKM-3, IC-39340-1, China mung and LM-1668 were collected from Department of Genetics and Plant Breeding, UAS, GKVK, Bengaluru and were used in the present investigation for *Agrobacterium* mediated transformation.

**Explant and media:** The regeneration protocol and media composition (Soumya, 2018, unpublished data) was followed in this study. The seeds were surface sterilized by initial washing under running tap water for 5-10 min, then washed with Tween-20 for 2 min followed by thorough wash with distilled water to remove soap solution. Further steps were conducted under aseptic conditions in Laminar Air Flow chamber (Clean Air Systems, India). The seeds were washed with 70 per cent ethanol solution for 3 min and then washed with 0.1 per cent of mercuric chloride solution for 2 min followed by 5-6 washes with sterile double distilled water and inoculated on half MS media (HiMedia, Laboratories, India) for 5-6 days, placed in a growth chamber (Fig. 4a) were used as the explant source for the single cotyledonary nodes (~1cm) for *Agrobacterium* co-cultivation. The shooting media (SM) supplemented with 5 $mgl^{-1}$ BAP, 50 $mgl^{-1}$ hygromycin (Muthukumar *et al*., 1996; Olhoft *et al*., 2003) and 250 $mgl^{-1}$ cefotaxime worked for the regeneration of multiple shoots. The regenerated shoots were continuously sub-cultured over the SM with an interval of 10-12 days under the same conditions, *Agrobacterium* contamination was avoided by cefotaxime wash (250 $mgl^{-1}$) of the explants.

The regenerated shoots recovered from selection media were transferred to shoot elongation media (SEM) supplemented with 0.3 $mgl^{-1}$ zeatin and 2.5 $mgl^{-1}$ 6-Benzylaminopurine (BAP) for shoot elongation and elongated shoots were sub-cultured on rooting media (RM) comprising of half MS with 0.2 $mgl^{-1}$ Naphthalene acetic acid (NAA). The entire experiment of *in vitro* regeneration was conducted under aseptic tissue culture facility with temperature of 24°C ±1°C, RH of 50-60 per cent under fluorescent and cool lighting in a 16/8h (light/dark) photoperiod and all growth hormones used were from Sigma-Adrich, USA.

**Infection and co-cultivation:** Transformed *Agrobacterium* were grown in yeast extract peptone (YEP) medium with rifamycin 100 $mgl^{-1}$ and 50 $mgl^{-1}$ kanamycin at 28 °C for 48 h. Bacterial cells were pelleted at a speed of 3500 rpm for 10 min and re-suspended in half MS solution plant culture medium at a density of $10^9$ cells per ml ($OD_{560}$=1) with 100 mM of acetosyrinzone and inoculated on the shoot regeneration media under dark condition for 48 h. (Bakshi *et al*., 2012).

**Molecular confirmation of putative transformants**

**Histochemical analysis of *gus* expression:** Transformants were assessed for *gus* expression by sampling a piece of leaf tissue and placing in GUS histochemical stain [50 mM sodium phosphate buffer ($Na_2HPO_4$ and $NaH_2PO_4$, pH 6.8), GUS solution (1mM X-gluc in DMFA and methanol)]. The sampled tissue was submerged GUS solution and incubation at 37 °C overnight. The tissues were bleached with absolute ethanol to remove the excess chlorophyll pigment and the tissues were observed under compound microscope (Jefferson *et al*., 1987)

**PCR amplification:** Genomic DNA of the transformed ($T_0$) plants were isolated by the modified SDS method (Islam *et al*., 2011; Swapnil and Peter, 2015) and subjected to PCR amplification *Rep* gene specific primers (FP: 5′ATGCCTAGACTCGGTCGTTTG3′ and RP: 5′TCAATTCGAGCGTCGAATTGC3′) and positive transformants were subjected to PCR amplification by vector specific *hptII* (*Hyg*$^{r)}$ gene specific primers (FP: 5′CTAGTTCTTTGCCCTCGGACGAGT3′ and RP 5′ATGAAAAAGCCTGAACTCACCGC3′) and *Vir* gene specific primers (FP: 5′GCCGACAGCACCCAGTTCAC3′ and RP: 5′CCTGCCGTAAGTTTCACCTCACC3′) all primers were procured from Bionova Lifesciences, India, PCR amplification of the isolated genomic DNA from putative transformants were performed using thermal cycler (Bio-Rad Laboratories, USA) PCR reactions were carried out with 20l reaction mixture containing 10x PCR buffer with 20 mM $MgCl_2$, 2.5mM dNTPs, deionized nuclease free water, forward and reverse primers, DNA template and 1U/µl *Taq* polymerase (BlackBio Biotech 3B DNA polymerase, India Ltd.).

The reaction conditions were as follows, 94°C for 5 min of initial denaturation followed by 35 cycles at 94 °C for 45s denaturation, annealing temperature of [( MYMV *Rep* gene specific primers, 59 °C for 1 min), (*nptII* gene specific primers 55°C for 1 min), (*Vir* gene specific primers 54 °C for 30s), (CaMV 35S promoter specific primers 54 °C for 30s)], extension temperatures of [( MYMV *Rep* gene specific primers, 72 °C for 1 min 45s ), (*nptII* gene specific primers 72 °C for 1 min), ( *Vir* gene specific primers 72 °C for

30s), (CaMV 35S promoter specific primers 72 °C for 30s)] and final extension at 72 °C for 7 min and hold. Amplified DNA fragments were then analyzed by electrophoresis using agarose gel 1 per cent and visualized after ethidium bromide staining (Sambrook *et al*., 1989) under UV gel documentation unit (Alpha InnonTech, USA).

## RESULTS AND DISCUSSION

### Isolation of MYMV *Rep* gene

The genomic DNA isolated from infected samples of mungbean were subjected to PCR amplification with the designed MYMV *Rep* gene specific primers and amplification revealed an expected size of approximately 1100 bp (~1.1kb) was obtained on 1 per cent agarose (Fig. 1a). Similar observations of the gene size of 1100 bp has been reported from Israel isolate and Bengaluru isolates respectively (Sunitha *et al*., 2013; Renuka *et al*., 2018), gene size of 1088 bp has been also reported (Tsai *et al*., 2013) from Pakistan isolate and 1188 bp (Reddy *et al*., 2011)

### Construction of pCAMBIA1305.2::CaMV35s::MYMV *Rep*::nos

The plasmid DNA (pTZ57RT::MYMV *Rep*) from the transformed *E.coli* DH5á cell was isolated and the pTZ57R/T plasmid containing *Rep* gene was confirmed by blue- white colony screening (Fig. 1b) and using the MYMV *Rep* gene specific primers. The PCR amplification by MYMV *Rep* gene specific primers showed the presence of amplicon size of 4˜ 1100 bp and also amplified by M13 primers and analyzed using NCBI-BLAST, confirmed it as MYMV *Rep* gene (NCBI accession number: MT863331) further matched with the other deposited MYMV sequences. According to sequencing results the orientation of MYMV *Rep* gene was confirmed and analysis by restriction digestion with SacI-HF and HindIII-HF indicated a release of MYMV (Fig. 1c) confirming the presence of *Rep* gene.

The positive pTZ57RT::MYMV *Rep* was used to obtain the release of MYMV *Rep* gene by restriction digestion with SacI-HF and HindIII-HF, simultaneously the empty vector pCAMBIA1305.2 was also cleaved at the respective sites. The release and digested binary vector was eluted and subjected to ligation to develop a construct pCAMBIA1305.2::CaMV35s::MYMV *Rep*::nos. Construct was transformed into *E.coli* DH5á cell and confirmed by colony PCR and restriction analysis, the amplified fragment and release of ~1100 bp was observed by electrophoresis on 1 per cent agarose gel (Fig. 2b and 2c).

The plasmid construct pCAMBIA1305.2:: CaMV35s::MYMV *Rep*::nos from transformed *E.coli* DH5α colonies was transformed into *Agrobacterium* cells and incubated over YEP media containing 50 mg $L^{-1}$ kanamycin and 100 mg $L^{-1}$ rifamycin, after incubation for 48h at 28 $^{o}$C, contruct isolated were subjected to colony PCR and the results revealed amplicon size of ~1100 bp confirmed it as MYMV *Rep* gene.

### Development of transgenic mungbean with MYMV *Rep* gene

The cotyledonary nodal explants derived from the seeds of four different varieties were subjected to infection with the *Agrobacterium* transformants containing pCAMBIA1305.2::CaMV35s::MYMV *Rep*::Nos construct and cultured on SM (Fig. 4b), after 12-15 days of inoculation the shoot buds were initiated (Fig. 4c), similarly cotyledonary nodal tissues were used for *Agrobacterium* mediated transformation studies (Krishanamurthy *et al*., 2000; Sharma and Anjaiah, 2000; Srinivasan *et al*., 2004; Mahalakshmi *et al*., 2006; Saini and Jaiwal, 2007; Chaudhary *et al*., 2007). The transformed $T_o$ shoots were selected on SM. On sub-culturing on SM, proliferation of multiple shoots and an average of 3-4 shoots were observed in all varieties (Fig. 4d) These results were consistent with the findings of previous reports in mungbean (Avenido and Hattori, 2001; Mundhara and Rashid, 2006; Yadav *et al*., 2010; Rout *et al*., 2020). During shoot proliferation many number of plantlets were lost due to contamination and rest of plantlets which survived (7 in number: KKM-7-MAC-14, KKM-8-MAC-14, IC-1-MAC-14, KKM-2-MAC-19, LM-8-MAC-19, CM-11-MAC-17 and CM-4-MAC-19), were transferred to SEM, The multiple shoots were elongated on SEM and elongation of shoots was observed. Induction of healthy root system is important for the regenerated plants, for root induction elongated shoots were sub-cultured on RM and the well-developed roots were observed after 2 weeks of root induction (Fig. 4f and 4g), similarly rooting on half MS with different auxin concentrations was reported by Geetha *et al*., 1998; Das *et al*., 2002; Vijayan, 2006; Mony *et al*., 2010 and Soumya, 2018, (unpublished data).

**Molecular analysis of putative transformants**

**GUS assay:** Transgenic plant production has been intimately connected to the β-*glucuronidase* (uidA or GUS) gene used as a reporter marker. The enzyme stability and the high sensitivity and amenability of the GUS assay to qualitative (histochemical assay) and to quantitative (fluorometric or spectrophotometric assay) detection are some of the reasons that explain the extensive use of uidA gene in plant genetic transformation. Hence it is a reliable method for screening transformed plants (Cervera, 2005).

The tissues were sectioned and observed under compound microscope (Jefferson *et al.*, 1987) Blue coloration was observed in leaf tissue sections of transformed which were positive for GUS assay and negative for control plants as there was no coloration (Fig. 5d). Similarly, transient and constitutive GUS expression was observed in different tissues of $T_0$ plants (Mekala *et al.*, 2016; Sahoo *et al.*, 2016; Baloda *et al.*, 2017; Bhajan *et al.*, 2019; Rout *et al.*, 2020).

**PCR analysis:** Putative transformants which survived after shoot elongation and with successful rooting were initially screened by *Rep* gene specific PCR amplification and out of 7 plants, 4 plants were confirmed as positive; KKM-7-MAC-14, KKM-8-MAC-14, KKM-2-MAC-19 and CM-11-MAC-17 for the presence of MYMV *Rep* gene, ~1100 bp was observed by electrophoresis on 1 per cent agarose gel (Fig. 5a). Later the PCR amplification of the four positive putative transformants was done by vector specific *hptII* (*Hyg*$^r$), which revealed the fragment size of ~1.2 kb over 1 per cent agarose gel electrophoresis (Fig. 5b). *Vir* gene specific primer amplification was not observed, which revealed that there was no *Agrobacterium* contamination in the apoplasts of $T_o$ transformants (Fig. 5c). According to the results obtained the KKM variety has good survivability and transformation efficiency with respect to *Agrobacterium* mediated transformation technique. Stable integration of *nptII* genes in the putative transformed shoots were confirmed through PCR analysis using *hptII* and *nptII* gene specific primers (Baloda *et al.*, 2017) and only *nptII* gene specific primers in transformed plants (Sahoo *et al.*, 2016; Bhajan *et al.*, 2019; Rout *et al.*, 2020).

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