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Research Article

Construction of RNAi Constructs for Soybean to Acquire Resistance against Mungbean Yellow Mosaic India Virus

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ABSTRACT:
As soybean belongs to legume family, it acts as a predominant source for vegetable protein to human diet. Moreover consumption of soybean improved the health of an individual and provides protection against various chronic diseases which had been already demonstrated by various studies. Hence, improving the quality and productivity of soybean has always been an imperative goal for scientists. Still, every year substantial losses in soybean production occur due to pathogen attack particularly, mungbean yellow mosaic India virus. Yellow mosaic disease became a major threat by affecting the yield loss of around $ 300 million dollars in various legume crops and in soybean, the yield loss has been estimated about 196.1 million tons. RNAi technology opens the possibility to raise considerable resistance against viral diseases in major crops. In the present study, we have constructed hairpin loop RNAi vectors which targeting Rep and IR gene of mungbean yellow mosaic India virus. The targeted genes were cloned in sense and antisense orientation initially in cloning vector pHANNIBAL subsequently subcloned in plant transformation binary vector pCAMBIA1301 under the control of 35S promoter and nos terminator. For plant transformation studies, the constructs were mobilized in to *Agrobacteriun tumafaciens* strain EHA105 which would be used to raise transgenic soybean plants having resistant against MYMIV.

Keywords: *RNAi, Yellow mosaic disease, Soybean, Mungbean yellow mosaic India virus, Rep, IR.*

[1] INTRODUCTION

Soybean is one of the most considerable important agriculture crops and having a much attention because of its high value nutritious content and beneficial compounds. As it belongs to legume family, soybean offers a rich source of vegetable protein to the human diet. In addition to protein, soybean is a major source of oil, carbohydrates, isoflavones, and minerals for human and animal nutrition since about one-third of the world's edible oils (30% of the consumption in the worldwide market) and two-thirds of protein meal are derived from soybean seed. Soybean protein content is well balanced compared to other protein sources. Moreover, Soybeans are a good source of minerals, B vitamins, folic acid and palmitic, stearic acids, unsaturated fatty acids, oleic, linoleic, and linolenic acids which make up 85% of the oil. Thus, soybean became one of the major world crops, occupying large acreages of land and the world production of soybeans has rising from about 70 million metric tons to over 200 million

metric tons which was tripled in last 20 years (www.soystats.com). About 80% of the soybeans are produced in North and South America. The United States, Brazil, and Argentina are the major soybean-producing countries followed by China and India. In recent years, soybeans have also been under intensive study because of their multiple health-enhancing properties. Although soybean breeding programs have developed varieties with improved yields, quality and disease resistance, every year substantial losses in soybean production still occur due to pathogen attack. Therefore, the understanding of pathogenesis of disease causing organisms is important not only to increase crop yield, but also to engineer disease resistant varieties since soybean is more susceptible to aphids and viruses.

Yellow mosaic disease caused by *Mungbean yellow mosaic virus* (MYMV) and *Mungbean yellow mosaic India virus* (MYMIV), belonging to the genus *Begomovirus* of the family *Geminiviridae* became a major threat by affecting the yield loss of around $ 300 million dollars [1] in various legume crops such as *Vigna radiata*, *V. mungo*, *Cajanus cajan*, *Gycine max*, *Vigna aconitifolia* and *Phaseolus vulgaris* [2]. In soybean, the yield loss has been estimated about 196.1 million tons [3].

Though various strategies available to acquire resistant against viral diseases, non-coding viral RNA had became effective tool followed by the introduction of RNAi mediated resistance [4]. RNA interference (RNAi) is a conserved sequence-specific, gene-silencing mechanism that is induced by double-stranded RNA [5]. Resistance obtained by silencing signals which can be amplified and transmitted between cells, and can be self-regulated by feedback mechanisms [6]. High efficient and specific suppression of targeted genes mediated by RNAi strategy has been widely adapted in plant virus disease engineering technology. In the present study, we aimed to construct RNAi vectors targeting Rep (replication initiation protein) gene and IR (intergenic region) gene of yellow mosaic virus to develop virus resistance in transgenic soybean plants.

[2] MATERIALS AND METHODS

Whole genome components of *Mungbean Yellow Mosaic India Virus* infecting soybean (MYMIV-Sb) were cloned and sequenced (size 2.7 kb and accession no.AYO49772) earlier in Plant Virology Unit, Division of Plant Pathology, and IARI. Replication initiation protein gene and intergenic region sequences were isolated from soybean isolate (MYMIV-Sb) DNA-A. In the Rep open reading frame, the segment of 1,760 nt co-ordinate to 2,266 nt was targeted for plasmid construction. For IR region from the nucleotide co-ordinate 2,730 nt to 182 was targeted. Primers were designed to get these fragments in sense and antisense orientation. The sense fragments were generated with *EcoR*I/*Xho*I restriction and the antisense fragments had *Hind*III/*Xba*I sites. The details of the primers are given in Table 1.

100 ng of plasmid DNA of MYMIV-Sb was subjected to PCR amplification with primers designed based on the sequence of MYMIV-Sb that span the targeted region in sense and antisense direction.

The PCR reaction contains 100 ng of plasmid DNA, 0.2 mM dNTPs, 1.0 U of *Taq* DNA polymerase (Sigma Genosys, Texas, USA), 0.4 µM of each primer and 2.5 µl of 10X Taq buffer in a total of 25 µl reaction. PCR amplifications were carried out in a PTC-200TM thermal cycler (MJ research Inc, Waltham, Mass, USA) programmed with one cycle of initial denaturation of DNA at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min (for denaturation), 55°C for 1 min (for annealing) and 72°C for 1 min (for extension), followed by a final extension at 72°C for 7 min. The amplified fragments for Rep 500 bp and IR 190 bp respectively were analyzed by electrophoresis at 100 V for 45 min in a 1% (w/v) agarose (Sigma, St. Louis, USA) gel containing 0.5 µg ml^{-1} ethidium bromide (Sigma, St. Louis, USA), and visualized on UV transilluminator .

Primer ID	Sequence (5' to 3')
Sense Rep Forward	TC**CTCGAG**GATCAGCTAGAGGAGG
Sense Rep Reverse	GC**GAATTC**GCTTCCCGTACTTGACG
Antisense Rep Forward	GC**AAGCTT**GCTTCCCGTACTTGACG
Antisense Rep Reverse	TC**TCTAGA**GATCAGCTAGAGGAGG
Sense IR Forward	TA**CTCGAG**TACTAAAAGCCTCTAGGG
Sense IR Reverse	TT**GAATTC**AAAGTCGTTCAACAATGG
Antisense IR Forward	TT**AAGCTT**AAAGTCGTTCAACAATGG
Antisense IR Reverse	TA**TCTAGA**TACTAAAAGCCTCTAGGG

Table: 1. Primers designed for Rep and IR hairpin MYMIV construct.

The PCR fragments were purified and restrict with respective enzymes *Hind*III/*Xba*I for Rep-antisense and *Xho*I/*Eco*RI for Rep-sense sequentially. For IR construct, PCR fragments were restricted with *Hind* III/*Xba*I for IR-antisense and *Xho1*/*Eco*RI for IR-sense. PCR restricted products were purified and kept for ligation. The cloning vector pHannibal was restricted with respective enzymes as mentioned above for each construct sequentially. After restriction, vector DNA was purified and ligated. After ligation, product was transformed into *E. coli* DH5α cells. The transformants were selected on Luria agar medium supplemented with ampicillin/nalidixic acid/X-gal/IPTG. The recombinant clones were observed, plasmid DNA were isolated and analyzed for the presence of the inserts by restriction digestion with *Xho*I/*Eco*RI restriction enzymes. After successful cloning of the fragments in sense orientation, the selected recombinants (pHANNIBAL/Rep-sense clone and pHANNIBAL/IR-sense clone) were restricted with the restriction enzymes *Hind*III/ *Xba*I for Rep and IR fragments (separately) cloning in antisense orientation. The Rep and IR region (500 bp and 190 bp) fragment in antisense orientation was amplified and the PCR amplicons were purified and restrict with the same enzymes *Hind*III/*Xba*I. The restricted vector and insert were ligated and transformed. The selected clones plasmid DNA was restricted with *Hind*III/*Xba*I which release Rep (500 bp) and IR (190 bp).

The Rep and IR constructs were released by restriction with NotI enzyme from pHANNIBAL cloning vector for the cloning in binary vector. The Rep and IR gene cassettes separately were transferred to the binary vector pGreenII 3304. pGreenII 3304 is restricted with *Not1* enzyme, followed by calf intestine alkaline phosphate treatment and purified for ligation. After ligation, transformed clones were confirmed by *XbaI* restriction enzyme. The derived construct was tried to mobilize into *Agrobacterium tumefaciens* strain LBA4404 for further transformation using pSoup as helper plasmid but *Agro* mobilization was not taking place. Thus, Rep and IR gene constructs were further transferred from pGreenII 3304 to pCAMBIA 1301. For cloning in pCAMBIA 1301 the Rep gene constructs were restricted with *Bam H1* and *Sac1* form pGreenII 3304 as same pCambia 1301 binary vector also restricted with *Bam H1* and *Sac1*, followed by calf intestine alkaline phosphate treatment and purified for ligation. After ligation transformed cloned were confirmed by *Bam H1*and *SacI* restriction enzyme.

The IR gene, pCAMBIA1301 binary vector and pGreenII 3304 IR gene constructs were restricted with *Sal1* and *Sac1* followed by calf intestine alkaline phosphate treatment and purified for ligation. After ligation transformed cloned were confirmed by restriction enzyme *Sal1* and Sac1. The constructs were mobilized into *Agrobacterium tumefaciens* EHA105 for further transformation using pRK2013 as helper plasmid. *Agrobacterium* containing Rep and IR constructs separately were selected against rifampicilin 10 µg ml^{-1} and kanamycin 50 µg ml^{-1}. The distinct colonies that appeared were confirmed through colony PCR by using sense and antisense primers for Rep and IR.

[3] RESULTS AND DISCUSSION

The inverted repeat region against the target gene forms a dsRNA structure which serves as the substrate of Dicer. Thus the sequences and length of the stem region directly affect RNAi efficiency [7]. Hence selecting target region play

an indispensable step for maximum silencing effect. In the present study, target genes were identified by multiple allignment which believed to have high efficiency in gene silencing. Primers with restriction enzymes amplified 500 bp of Rep and 190 bp of IR genes in PCR analysis (Fig. 1).

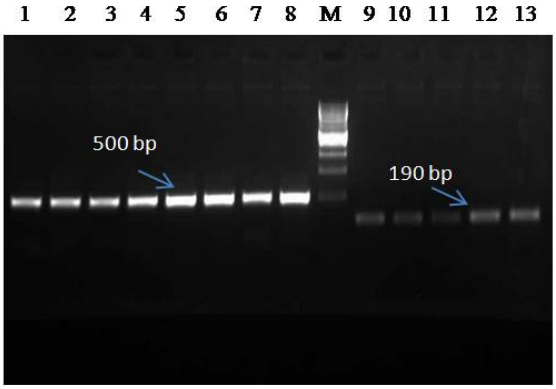

Fig: 1. Amplification of Rep sense (~500bp) and IR fragments in sense (~190bp) orientation from MYMIV-Sb.Lane 1-8: Rep; Lane M: ladder; Lane 9-13: Intergenic Region.

When these amplified products were ligated into pHannibal vector, the selected transformed colonies produced expected size of amplicons and released respective fragments from plasmid when digested with restriction enzymes *Hind*III/*Xba*I and *Xho1/ EcoRI* and *Hind III/ XbaI* and *Xho1/EcoRI* for Rep and IR sense and antisense genes, respectively. Among ten colonies screened, four colonies and one colony showed positive restriction digestion pattern for Rep and IR cloning, respectively (Fig. 2).

As stated by Wesley et al [8], intron-spliced hpRNA constructs appear to give a higher proportion of silenced transformants than intron-free hpRNA constructs since the process of intron-splicing aligns the arms of the hpRNA, facilitating their duplex formation in the spliceosome complex, whereas the arms of hpRNAs have to find their self-complementarity by random, but tethered, collisions. They also suggested that if there is a threshold of duplex RNA required for PTGS in plants, and then facilitating more efficient duplex RNA formation from ihpRNA might raise the level in low transgene-expressing plants such that PTGS is enabled. Thus the enhanced RNAi effect can be often observed by using an intron spacer rather than the GUS or GFP spacers [8].

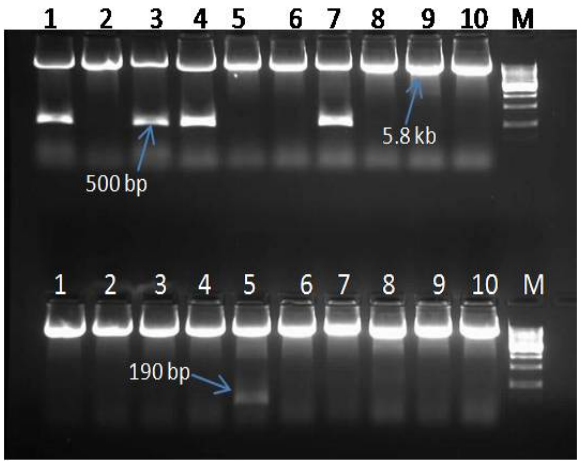

Fig: 2. Restriction of recombinant clones by respective enzymes for Rep and IR fragments in pHANNIBAL cloning vector. Upper band is pHANNIBAL vector 5.8 kb, lower band is Rep 500 bp and IR 190 bp.

For conventional cloning, pHANNIBAL is the most employed vector in which the PCR fragments of the target gene are produced by using primers with restriction sites, and cloned successively into both upstream and downstream region of the spacer to become the two arms of the hairpin construct [7].The high incidence and degree of silencing in plants transformed with pHANNIBAL constructs suggest that it could form the basis of a high-throughput silencing vector [8] as observed in the present study.

The subsequent cloning in plant transformation vector pGreenII 3304 [9] with sense and antisense under the control of 35S promoter was successfully done by using Not1 enzyme (Fig 3). Among 8 colonies and 2 colonies screened, five numbers of colonies and two number of colonies showed positive digestion pattern for IR and Rep cloning, respectively (Fig. 4).

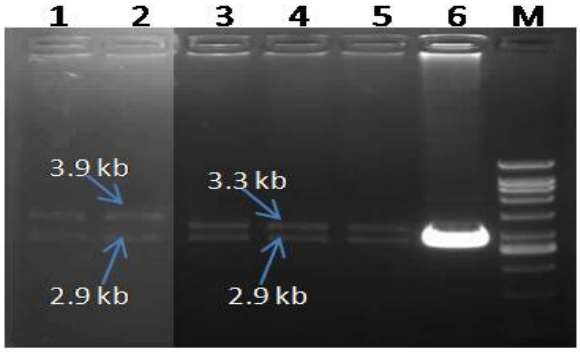

Fig: 3. Agarose gel electrophoresis of *Not*I restricted pHANNIBAL hp Rep and pHANNIBAL hp IR. Lane 1-2: Rep; Lane 3-5: IR; Lane 6: pHANNIBAL

plasmid restricted with *Not*I; Lane M: 1 kb DNA ladder.

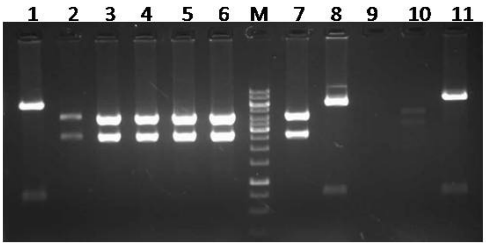

Fig: 4. Confirmation of Rep and IR RNAi construct with 35S promoter and OCS terminator in pGreenII 3304 by restriction digestion with *XbaI* enzyme. **Lane 1-8:** RNAi_IR constructs confirmed clones in which 2,3,4,5,6,7 are positive while **lanes 10 and 11** have RNAi_Rep constructs confirmed clone.

Since pGreenII 3304 vector could not co-operate with *A. tumefaciens* LBA 4404 strain, again the constructed vectors for both genes were sub-cloning into pCAMBIA 1301 successfully (Fig. 5 and 6).

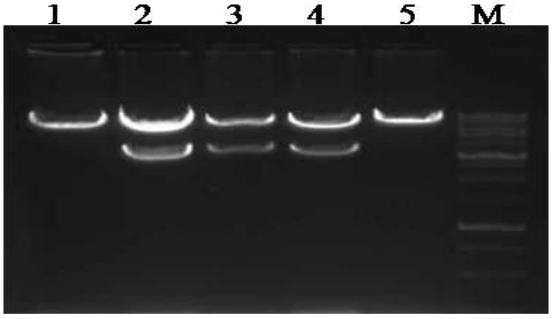

Fig:5. Restriction of pCambia1301/RNAi_Rep constructs clone by *BamH*I & *Sac*I enzymes for final confirmation, to get insert & vector separate. **Lane 1:** pCAMBIA1301 linerized vector; **Lanes 2-4:** Upper band vector and lower band Rep insert. Lane **2,3,4** showed positive result.

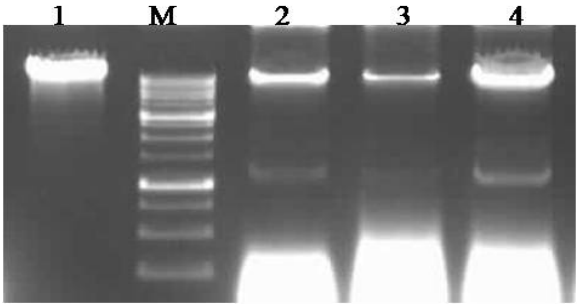

Fig: 6. pCAMBIA1301/RNAi_IR clone restricted by *Sal*I & *Sac*I enzyme to get insert & vector separated. **Lane 1:** pCAMBIA1301 linerized vector; **Lane M:** Marker, **Lanes 2-4:** Upper band vector and lower band IR insert.

Then they were subsequently mobilized into *Agrobacterium tumefaciens* EHA105 for further transformation using pRK2013 as helper plasmid. Confirmed colonies were used for inoculation studies and these constructs are also being in use for *Agro* transformation of soybean to develop the transgenic plants against MVMIV.

The schematic representation of RNAi constructs pCAMBIA1301-RNAi_REP and pCAMBIA1301-RNAi_IR which targeting the most conserved region of replication initiation protein gene and intergenic region are given in Fig. 7.

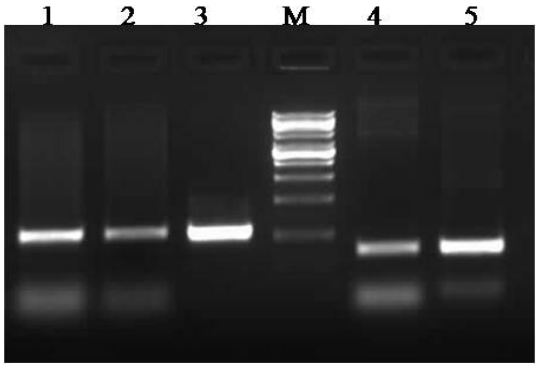

Fig: 6. Agarose gel electrophoresis of PCR amplicon of selected transconjugant colony of *A. tumefaciens* (strain LBA4404) having construct of pGrnhp using sense strand primers. Lane 1-3: PCR amplicon of sense strand of pGrnhpRep construct; Lane 4-5: PCR amplicon of sense strand of pGrnhpIR construct; Lane M: 1 kb DNA ladder;

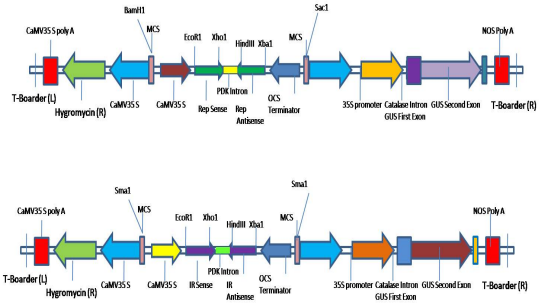

Fig: 7. Linear map of the RNAi constructs in vector pCambia 1301 present within the *Agrobacterium tumefaciens* strain EHA 105. A: RNAi_Rep ,B: RNAi_IR

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