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# Gene Stacking for Fungal Resistance in Plant Transformation Vector

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**Fungal diseases like early blight, late blight, fusarium wilt cause 30-40 per cent loss in fruit production. Form past decade many transgenic plants had been developed using genes encoding chitinases and glucanases with the objective of imparting fungal disease resistance. Since the genes encoding chitinase and glucanase act synergistically. The study was performed to construct plant transformation vector pRAGS carrying both *ech*42 and *bgn* under single T-DNA region. Initially, *Hind*III site at 5' end of earlier cloned *bgn* (*T. harzianum*) was removed using primers during reamplification of the gene. The amplicons were cloned into pTZ57R/T containing T overhangs at *Eco*321 site and transferred to *E. coli* DH5a and further to plant transformation vector pBI121 which was named as pRA121. In order to clone another gene (*ech*42) into pRA121, expression cassette from iHP vector was transferred to pRA121 and named as pRAG121. Further in order to gain *Xho*I site for cloning *ech*42 gene into pRAG121, *ech*42 (pSUM1) was cloned into pYES2/CT, named as pSAG1, *ech*42 from pSAG1 cloned with *Kpn*I and *Xho*I in pRAG121 and named as pRAGS121. The vector constructed in the present study can be used to transform important crop plants to have enhanced resistance to fungal diseases.**

**Keywords**: Gene stacking, Chitinase, Glucanase , Gene pyramiding.

Fungal diseases like early blight, late blight, fusarium wilt cause 30-40 per cent loss in fruit like tomato production (Punja 2006). Form past decade many transgenic plants had been developed using genes encoding chitinases and glucanases with the objective of imparting fungal disease resistance.

In-vivo study showed a collective protective interaction of the co-expressed anti-fungal proteins whereas class I tobacco chitinase and â-1, 3-glucanase acted synergistically. Class II chitinase combined with higher amounts of class I â-1, 3-glucanase showed confined antifungal activity in vitro (Sela-Buurlage *et al*., 1993). Transgenic wheat plants engineered with chitinase and b-1, 3-glucanase genes showed resistance to scab and powdery mildew (Bliffeld *et al.*, 1999) diseases. Transgenic *Brassica napus* lines transformed with barley chitinase and b-1, 3-glucanase genes showed improved resistance against *Leptosphaeria maculans* (Melander *et al.*, 2006).

A number of attempts such as sexual crossing of two different transgenic plants, sequential retransformation (Lapierre *et al.,* 1999), co-transformation with multiple plasmids (Chen

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*et al.,* 1998 and Hadi *et al.,* 1996) or with single plasmids on which several transgenes are linked (De Gray *et al.,* 2001; Goderis *et al.,* 2002; Akula *et al.,* 2011; Awah *et al.,* 2011; Erika *et al.,* 2013 and Sharad *et al.,* 2015) had been made to introduce multiple genes into plant genomes. These attempts has specific limitations: genetic crosses are time consuming, requirement of different selectable marker genes in sequential retransformation, the efficiency of co-transformation with multiple plasmids is inversely proportional to plasmid number and co-transformation with separate multiple plasmids is a rare event. Therefore it is hard to control their copy number and arrangements among transgenes. In addition, use of biolistics approach for multiple plasmid transformation leads to integration of genes into a few chromosome loci at high copy number, which is not favorable for expression of transgenes (Hadi *et al.,* 1996; Chen *et al.,* 1998; Gelvin *et al.,* 1998 and Maqbool *et al.,* 1999). Hence, co-transformation with linked transgenes in single vectors is a conventional and reliable approach.

## METHOD AND MATERIAL

**Construction of plant transformation vector carrying *bgn* and *ech*42 gene (*chit*42)**

**Removal of restriction sites in the 5' end of *bgn* through PCR**

The new forward primer was designed to exclude *Hind*III site at 5' end of the cloned gene using gene tool software and named as Modglu. Both forward and reverse primers specific to full length *bgn* were designed earlier (Mala, 2007). This modglu primer was used with the reverse primer to amplify *bgn* from pSGH2. The sequence of primer used is given below Z-Glu-6 Modglu Forward5'ATCAAGATGAAGTACACCATCG TTGCTCCG3' Reverse beta 1,6 *T. harzianum* 5'GCGCGGCCGCCAATCACTCGTGATTTACC 3'. The purified PCR products of 1.3 kb (50 ng/ ìl) were ligated to pTZ57R/T vector (2886 bp) as described in InsT/A clone™ PCR product cloning kit (#k1214) from MBI, fermentas USA. These circular plasmids with inserts of 1.3 kb were directly transferred to *E. coli* DH5a following the protocol mentioned by Sambrook and Russell (2001) with minor modifications. The clone was named as pSG1.

**Sub Cloning of *bgn* (restriction site modified) into plant transformation vector**

A plant transformation vector pBI121 was used for this purpose. pSG1 and pBI121 is restricted with *Xba*I and *Bam*HI. The insert of 1.6 kb size released from pSG1 was eluted and ligated with linearzed pBI121, transferred into *E. coli* DH5á. The clone was named as pRA121 (carrying CaMV35S: *bgn*: NOST cassette).

**Sub Cloning of plant expression cassette from iHP vector to pRA121**

The vector pRA121 and the clones iHP (provided by Dr. Dinesh Kumar, Directorate of Oil seeds Research, Hyderabad, INDIA) were isolated. Digestion of pRA121 and iHP was done with restriction enzymes *Hind*III. The insert of 1 kb size from iHP (carrying CaMV35S: *Catalaseintron*: PolyA ) was eluted ligated with linearized pBI121 and transferred into *E. coli* DH5á. The clone was named as pRAG121 (carrying both CaMV35S: *bgn*: NOST cassette; CaMV35S: *Catalaseintron*: PolyA).

**Sub Cloning of endochitanase genes into yeast expression vector**

In order to have *Xho*I restriction site from the MCS of pYES2/CT for cloning *ech*42 gene into pRAG121 previously cloned *ech*42 gene (Upendra, 2006) was first cloned into yeast expression vector (pYES2/CT). The vector pYES2/CT and the clones pSUM1C (carry *ech*42) were isolated and restricted with *Bam*HI and *Kpn*I. The insert of 1.6k b size was eluted from pSUM1C. Ligated with linearized pYES2/CT and transferred into *E. coli* DH5á. The colonies obtained were further streaked on Luria agar with ampicillin (100 ìg/ml). The clone was named as pSAG1.

**Sub Cloning of *ech*42 gene from pSAG1 vector to pRAG121**

A plant transformation vector pRAG121 carrying both CaMV35S: *bgn:* NOST cassette; CaMV35S: *Catalaseintron*: PolyA was used for this purpose. Sequential digestion of pRAG121 and pSAG1 was done with two restriction enzymes *Xho*I and *Kpn*I. The insert of 1.6 kb size released from pSAG1 was eluted. Ligated with linerized vector pRAGS121 and transferred into *E. coli* DH5á.Finaly the catalase intron is replaced by ech42 and the clone was named as pRAGS121 (carrying CaMV35S: *bgn*: NOST; CaMV35S:*ech*42: PolyA cassette)

## RESULTS AND DISCUSSION

**Construction of plant transformation vector carrying both *bgn* and *ech*42**

**Sub cloning of restriction site modified *bgn* (gln) from pSGH2**

Both the primers were used at 10 pM per il concentration. The template DNA of pSGH1 gave an amplicon of 1.3 kb was cloned to pTZ57R/T vector. Recombinant cells were selected based on blue/white colony assay. All of these showed the presence of 1.3 kb insert in PCR and restriction analysis (with *Xba*I and *Bam*HI enzymes). The PCR and restriction analysis of selected clones for sequencing is shown in Fig. 7 and Fig. 10 respectively. The clones were named as pSG1 and the vector map is shown in Fig. 2.

**Sub cloning of *bgn* (restriction site modified) into plant transformation vector pBI121**

The gene *bgn* is cloned into pBI121 from pSG1. Plasmid DNA isolated from the clones was confirmed through PCR (Fig. 7) and restriction analysis using *XbaI* and *Bam*HI enzyme (Fig. 10) and named as pRA121 clones. All of these showed the presence of 1.3 kb insert in PCR and restriction analysis (Fig. 3).

**Sub cloning of cassette from iHP vector to pRA121**

In order to have another plant expression cassette (CaMV35S: *Catalase intron*: PolyA) to clone *ech*42 into pRA121, iHP vector was restricted with *Hind*III to release expression cassate and was cloned in to pRA121. Plasmid DNA isolated from the clones was confirmed through restriction analysis using *Hind*III enzyme (Fig. 10) and named as pRAG121 (carrying both CaMV35S: *bgn:* NOST cassette; CaMV35S: *Catalase intron:* PolyA) clones (Fig. 4). All of these showed the presence of 980 bp insert in PCR and restriction analysis.

**Sub cloning of the *ech*42 into the yeast expression vector**

In order to have *Xho*I restriction site for cloning *ech*42 into pRAG1 previously cloned *ech*42 (Upendra, 2006) was first cloned into yeast expression vector (pYES2/CT). Plasmid DNA isolated from the clones was confirmed through PCR (Fig. 8) and restriction analysis using *Kpn*I and *Bam*HI enzyme (Fig. 10) and named as pSAGI clones (Fig. 5). All of these showed the presence of 1.3 kb insert in PCR (Plate 5) and 1.6 kb in restriction analysis (Plate 7).

**Sub cloning of *ech*42 from pSAG1 vector to pRAG121**

For the construction of a plant transformation vector carrying both *bgn* and *ech*42 from *Trichoderma*, final cloning was performed by inserting *ech*42 from pSAG1 vector into pRAG121. Plasmid DNA isolated from these clones was confirmed through PCR (Fig. 9) and restriction analysis using *Xho*I and *Kpn*I *enzyme* (Fig. 10) and named as pRAGS121 (CaMV35S: *bgn*: NOST; CaMV35S:*ech42*: PolyA cassette) clones (Fig. 6). All of these showed the presence of 1.3 kb insert in PCR and 1.6 kb in restriction analysis. Schematic diagram of vector development carrying both *ech*42 and b-1, 6-glucanase is shown in Fig. 1.

Co-transformation with multiple genes in single vectors is a conventional and reliable approach. However, this approach is technically demanding. Our motto of experiment is to construct plant transformation vector (pRAGS121) having both *bgn* and *ech*42 genes which act in synergy to degrade fungal cell wall under two separate expression cassette with in a single T-DNA region. On the same T- DNA genes are close to each other. They are tightly linked and will not segregate at higher rate. Further both the genes integrate at the same chromosomal location and will be together in the subsequent generation. Two or more genes, each with its own promoter and terminator, on the same T-DNA region will transfer as a single entity into a plant (i.e. on a single T-DNA for *Agrobacterium*-mediated transformation). This approach is considered as a special case of co-transformation. Now a day co-transformation with the linking 'effect' genes is a preferable strategy that has been used with great advance to stack genes in many GM crops and has gained regulatory approval. Although, a small number of genes (typically two) have been used in this study but many ambitious research projects have used larger linked-gene cassettes. For example, four or five genes (three or four genes related to PHB synthesis, plus a selectable marker gene) have been linked within one T-DNA and introduced into *Arabidopsis* (Bohmert *et al.*, 2000, 2002) or oilseed rape (Slater *et al.*, 1999). In case if four or more genes needed to be introduced, it is recommended to use several T-DNA cassettes of moderate size

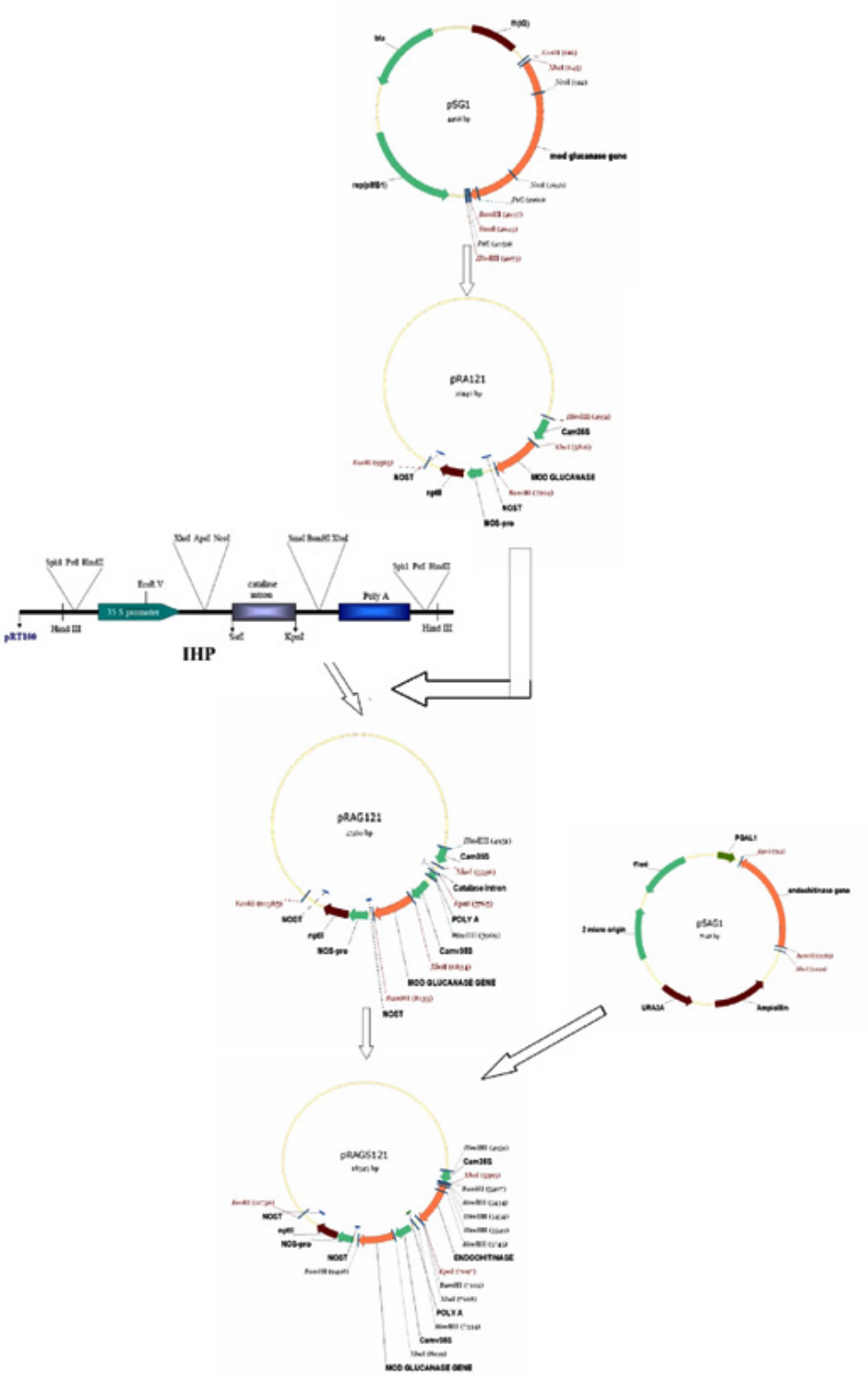


**Fig. 1.** Schematic diagram of vector construction carrying both *bgn* & *ech*42 gene.

(two or three linked 'effect' genes) in combination with the co-transformation of the different T-DNAs. Using this strategy, three genes for PHB biosynthesis, plus a selectable marker gene, were introduced on two T-DNAs into *Arabidopsis* and 16% of the resulting plants produced PHB (Poirier *et al.*, 2000). Similarly, 'Golden rice' developed by co-transformation of two T-DNAs, each consisting two linked genes (Ye *et al.*, 2000). Constructs containing as many as 10 foreign DNA fragments had also been created and the *Agrobacterium*-mediated transfer of 6 linked resistance genes was demonstrated in rice (Lin *et al.*, 2003). Sridevi *et al.*, (2008) used *Agrobacterium tumefaciens* LBA4404 (pSB1) harbouring the binary vector pNSP3 with rice chitinase and tobacco b-1, 3-glucanase genes (under ubiquitin and CaMV 35S promoter respectively) for transformation of elite indica rice variety Pusa Basmati 1 to enhance sheath blight resistance. Three genes encoding fungal cell wall degrading enzymes (CWDEs), *ech*42, *nag*70 and *gluc*78 from the biocontrol fungus *Trichoderma atroviride* were inserted into the binary vector pCAMBIA1305.2 singly and in all possible combinations and transformed to rice plants **(**Liu *et al.*, 2004**).** Even plasmid construct (pMOG539) carrying four-genes (class I and class II chitinases and /3-1, 3-glucanases) in a single T-DNA under separate CaMV35S promoter were made by assembly of the respective genes next to the neomycin phosphotransferase II (*npt*ll) gene


**Fig. 2.** Vector map of pSG1

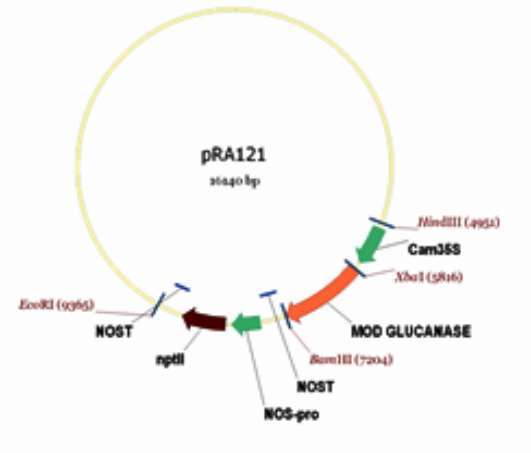


**Fig. 3.** Vector map of pRA121

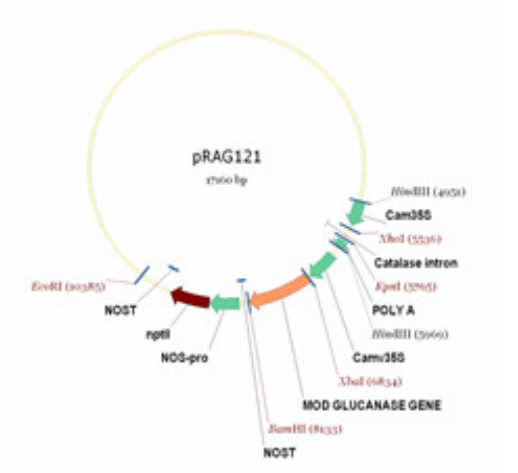


**Fig. 4.** Vector map of pRAG121

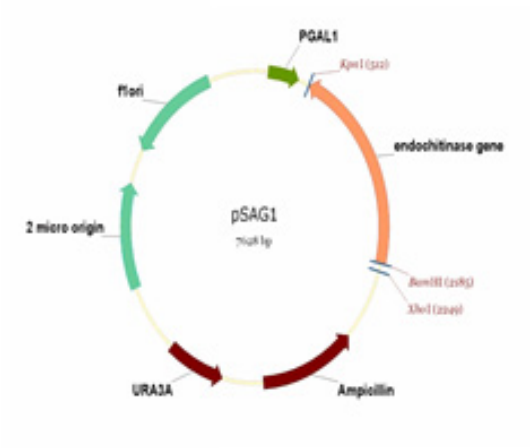


**Fig. 5.** Vector map of pSAG1

in the binary plasmid pMOG402 (Jongedijk *et al.,* 1995).

This strategy not only overcome the problem associated with segregation but also solves the problem of selection of multiple antibiotic markers in co-transformation with multiple plasmid which leads to poor regeneration due to high selection pressure and also it is tough to have many selectable marker which is not sensitive to crop like rice and tomato. Although the use of large number of selectable markers during multiple plasmid co-transformation is constantly increasing, that would encounter significant hurdles to regulatory approval and public acceptance.

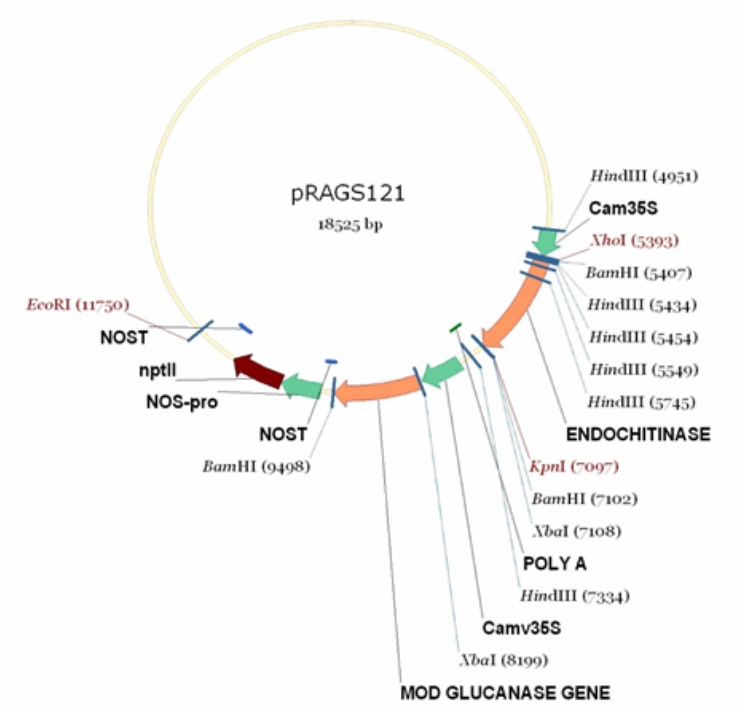


**Fig. 6.** Vector map of pRAGS121

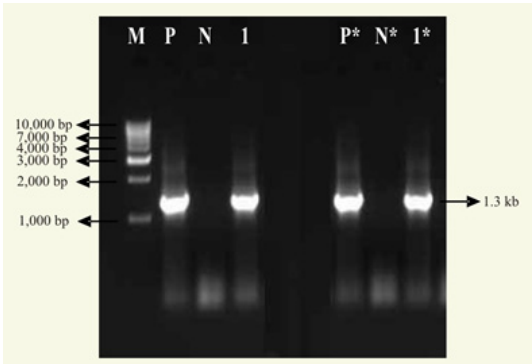


**Fig. 7.** PCR conformation of pSG1 & pRA121 clones. M : 1 kb ladder P: Positive control (plasmid of pSGH2), N: Negative control (without plasmid), 1: Amplification of b 1-6, glucanase gene in pSG1,P*: Positive control (plasmid of pSG1), N*: Negative control (without plasmid) and 1*: Amplification of b 1-6, glucanase gene in pRA121

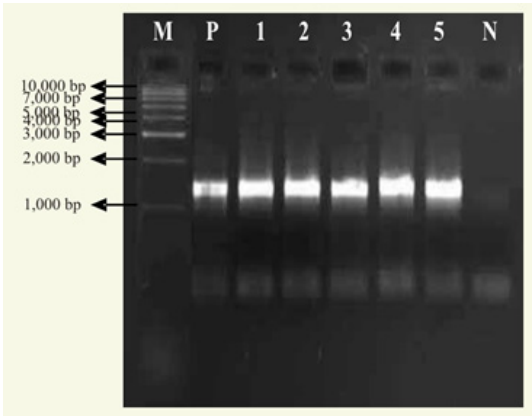


**Fig. 8.** PCR conformation of pSAG1 clones. M: 1 kb ladder, P: Positive control (plasmid of pSUM1C), 1-5: Amplification of endochitinase gene in pSAG1 andN: Negative control (without plasmid)

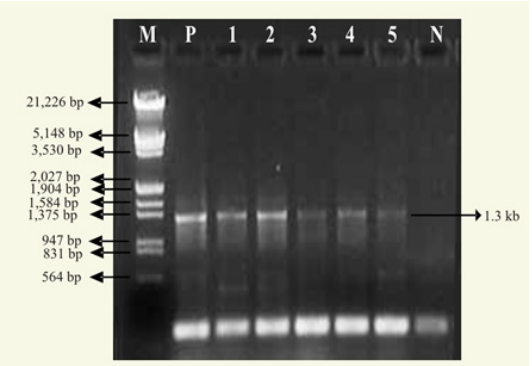


**Fig. 9.** PCR conformation of pRAGS121 clones. M: 1 kb ladder P: Positive control (plasmid of pSAG1), 1-5: Amplification of endochitinase gene in pRAGS121 and N: Negative control (without plasmid).

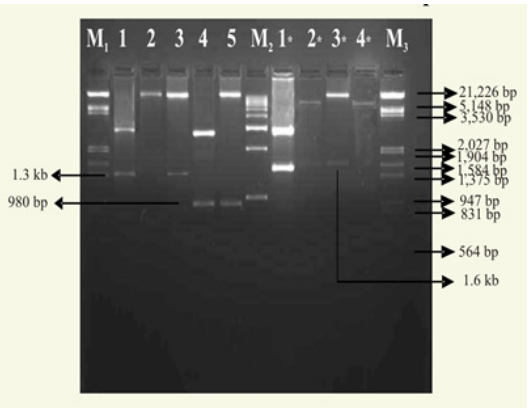


**Fig. 10.** Restriction conformation of pSG1, pRA121, pSAG1& pRAGS121. M1 & M3: l*Hind*III / *Eco*RI double digest ladder, 1: pSG1 cut with *Xba*I and *Bam*HI, 2: pBI121 cut with *Xba*I and *Bam*HI, 3: pRA121 cut with *Xba*I and *Bam*HI, 4: iHP cut with *Hind*III, 5: pRAG121 cut with *Hind*III, M2: 1 kb ladder 1*: pSAG1 cut with *Bam*HI and *Kpn*I, 2*: pRAGS121 cut with *Kpn*I and *Xho*I and 3*: pYES2/CT cut with *Bam*HI and *Kpn*I

One recent *Agrobacterium* mediated co-transformation experiment with multiple plasmids in *Arabidopsis found* that most inserts were very complex loci consisting of multiple tandem or inverted T-DNA repeats that often also included the complete binary vector sequence (Stuitje *et al*., 2003). Such integration patterns leads to transgene silencing in subsequent generations and uphold the regulatory approval of these transgenic crops. Hence, gene stacking with single plasmid in plant transformation vector can give ride of many hurdles coming in way of gene pyramiding in plants and proven to be the most favorable technique to transfer genes which act in synergistic manner.

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