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 ech42 and bgn under single T-DNA region. Initially, HindIII 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 Eco321 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 (ech42) into pRA121, expression cassette from iHP vector was transferred to pRA121 and named as pRAG121. Further in order to gain XhoI site for cloning ech42 gene into pRAG121, ech42 (pSUM1) was cloned into pYES2/CT, named as pSAG1, ech42 from pSAG1 cloned with KpnI and XhoI 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 ß-1, 3-glucanase acted synergistically. Class II chitinase combined with higher amounts of class I ß-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...
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 ech42 gene (chit42)**

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

The new forward primer was designed to exclude *HindIII* 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′ATCAAGATGAAAGTACACCATCG TTGCTCCG3′ Reverse beta 1,6 T. harzianum 5′GCCGCGGCCGCAAATCACTCGGTATTACC 3′. The purified PCR products of 1.3 kb (50 ng/ l) were ligated to pTZ57R/T vector (2886 bp) as described in InsT/A clone™ PCR product cloning kit (yk1214) 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 *XbaI* and *BamHI*. The insert of 1.6 kb size released from pSG1 was eluted and ligated with linearized pBI121, transferred into *E. coli* DH5a. 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 *HindIII*. The insert of 1 kb size from iHP (carrying CaMV35S: *Catalase intron*; PolyA ) was eluted ligated with linearized pBI121 and transferred into *E. coli* DH5a. The clone was named as pRAG121 (carrying both CaMV35S: bgn: NOST cassette; CaMV35S: *Catalase intron*: PolyA).

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

In order to have *XhoI* restriction site from the MCS of pYES2/CT for cloning ech42 gene into pRAG121 previously cloned ech42 gene (Upendra, 2006) was first cloned into yeast expression vector pYES2/CT). The vector pYES2/CT and the clones pSUM1C (carry ech42) were isolated and restricted with *BamHI* and *KpnI*. The insert of 1.6k b size was eluted from pSUM1C. Ligated with linearized pYES2/CT and transferred into *E. coli* DH5a. The colonies obtained were further streaked on Luria agar with ampicillin (100 ig/ml). The clone was named as pSAG1.

**Sub Cloning of ech42 gene from pSAG1 vector to pRAG121**

A plant transformation vector pRAG121 carrying both CaMV35S: bgn: NOST cassette; CaMV35S: *Catalase intron*; PolyA was used for this purpose. Sequential digestion of pRAG121 and pSAG1 was done with two restriction enzymes *XhoI* and *KpnI*. The insert of 1.6 kb size released from pSAG1 was eluted. Ligated with linearized vector pRAGS121 and transferred into *E. coli* DH5a. Finally the catalase intron is replaced by ech42 and the clone was named as pRAGS121 (carrying CaMV35S: bgn: NOST; CaMV35S: ech42: PolyA cassette)
RESULTS AND DISCUSSION

Construction of plant transformation vector carrying both bgn and ech42

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

Both the primers were used at 10 pM per µl 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 XbaI and BamHI 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 and restriction analysis (Fig. 9) and restriction analysis using XhoI and KpnI 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 ech42 into pRA121, iHP vector was restricted with HindIII to release expression cassette and was cloned in to pRA121. Plasmid DNA isolated from the clones was confirmed through restriction analysis using HindIII enzyme (Fig. 10) and named as pRAG121 (carrying both CaMV35S: bgn: NOST; CaMV35S: ech42: PolyA cassette) clones (Fig. 4). All of these showed the presence of 980 bp insert in PCR and restriction analysis.

Sub cloning of the ech42 into the yeast expression vector

In order to have XhoI restriction site for cloning ech42 into pRAG1 previously cloned ech42 (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 KpnI and BamHI 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 ech42 from pSAG1 vector to pRAG121

For the construction of a plant transformation vector carrying both bgn and ech42 from Trichoderma, final cloning was performed by inserting ech42 from pSAG1 vector into pRAG121. Plasmid DNA isolated from these clones was confirmed through PCR (Fig. 9) and restriction analysis using XhoI and KpnI 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 ech42 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 ech42 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 hgn & ech42 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), ech42, nag70 and gluc78 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 (nptII) gene

![Fig. 2. Vector map of pSG1](image1)

![Fig. 3. Vector map of pRA121](image2)

![Fig. 4. Vector map of pRAG121](image3)

![Fig. 5. Vector map of pSAG1](image4)
in the binary plasmid pMOG402 (Jongedijk et al., 1995). This strategy not only overcomes 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 and N: Negative control (without plasmid)
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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REFERENCES


Evaluation of multiple defense responses in transgenic plants.


