639

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Construction of a recombinant vector for constitutive expression of Hevea chitinase in *Bacillus subtilis*, an endosymbiont of *Hevea brasiliensis*

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INTRODUCTION

Recent studies indicate the presence of bacterial endophytes (*Bacillus subtilis*, strain 8LK) in the intercellular spaces of *Hevea brasiliensis*. *B. subtilis* which is generally regarded as a safe organism is involved in controlling plant pathogens (Sturz & Matheson, 1996), imparting drought tolerance and promoting growth and development in plants. Bacterial endophytes which are known as bio control agents (Berg *et al.*, 2005), if genetically modified to over-express gene of interest can impart stress tolerance in the tissues of *Hevea*. Hence in this study, an attempt was made to construct a recombinant expression secretion vector with an aim to over-express chitinase gene of *Hevea* (which can control the growth of fungal mycelium of leaf fall disease causing *Corynespora cassicola*) in *B. subtilis*. The strategies adopted and the steps involved in the construction of the *Bacillus* specific expression secretion vector and the results obtained are explained in this paper.

MATERIALS AND METHODS

The leaves of disease tolerant clone GT1, were infected with the fungus, *Corynespora cassiicola* using standard procedure. mRNA was isolated using Dyna beads (Invitrogen, USA) and cDNA was synthesized using Superscript III™ RT First strand synthesis kit, Invitrogen, USA. PCR amplification of chitinase gene was performed with standard conditions using specific primers flanked with restriction sites (*Bam*H I in the forward primer and *Xba* I in the reverse primer) to facilitate directional cloning. pGEMT Easy vector was used for cloning the purified PCR amplicon. Colony PCR and restriction digestion analysis were performed as per the standard protocol. DNA sequencing was carried out by Macrogen, Korea. A *Bacillus* specific, pHT43 expression secretion vector (Mobitec Germany) was used for the protein production. The pHT43 vector was modified by removing the *lac*I repressor gene using *Apa* 1 and *Sac* 1 restriction enzymes to avoid induction in field conditions. A recombinant vector was constructed using the modified vector and purified chitinase gene. A mutated strain of *Bacillus subtilis*, WB8000N which is specifically modified for the expression sectretion vector pHT43, was purchased from Mobitec, Germany can be used as a positive control for transformation and expression studies. Both the strains, endophytic *Bacillus subtilis* and the mutated strain, WB800N were transformed with a protocol already standardized in this work and was confirmed by colony PCR and slot lysis. From the mutated strain, protein was extracted using acetone precipitation method. SDS PAGE gel was used for the protein visualization.

RESULTS AND CONCLUSIONS

cDNA, synthesized from mRNA (Fig.1) of the infected leaves (of *Hevea)* was used as template DNA to PCR amplify (Fig. 2), the 978 bp coding region of chitinase gene. The transformants of pGEMT Easy

Fig.1. mRNA isolated from infected leaves

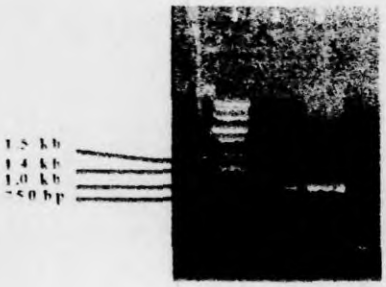


Fig.2. Chitinase gene from cDNA of *Hevea*

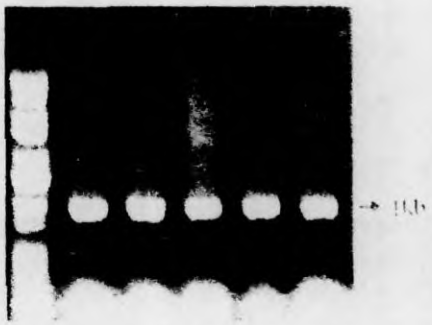


Fig.3. Colony PCR of *E. coli* pGEMT/chitinase

Lane1: Wide range marker
Lane2:singledigestion*Xba* I
Lane3:Single digestion*Bam*H I
Lane4&5:Double digestion *Xba* I and *Bam*H I
Lane6:Uncut pGEMT/chitinase

Fig. 4. Restriction digestion of p GEMT/chitinase

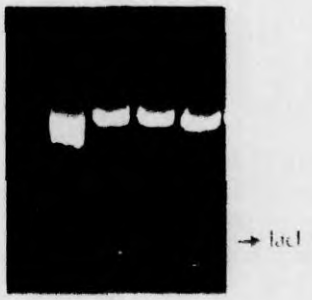
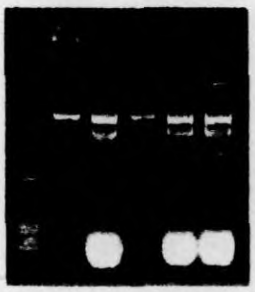


Fig. 5. Restriction digestion of pHT43 to release *lac*I



Lane1: Wide range marker
Lane2: Positive control
Lane3:Uncut pHT43 lacI
Lane4:Single digestion*Sam*HI
Lane5:Single digestion *Apa*I
Lane6:Single digestion *Sac*I

Fig.6. Confirmation of modification of pHT43

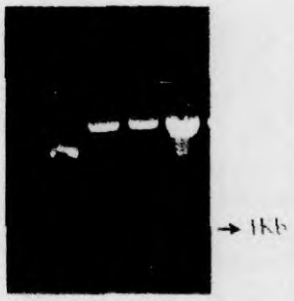


Fig.7. Restriction digestion of the recombinant vector

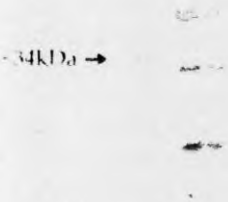


Fig. 8 SDS PAGE

Lane1&2: Protein molecular weight marker (low range) Lane 3: Epression of chitinase from *B. subtilis* Lane4: negative control

Fig. 9 Chitinase activity

X axis: Time in hour after reaching log phase. Y axis: Unit/ml of chitinase activity. Blue: *Bacillus subtilis* with modified pHT43 (Negative control). Yellow: *Bacillus subtilis* with recombinant vector (Test Sample)

Fig. 10. Colony PCR of transformed endophytic *B. subtilis*

vector,chitinase (purified) were selected based on amplification of 1 kb band of chitinase gene in colony PCR (Fig.3) and release of the fragment from plasmid in restriction digestion analysis (Fig.4). When the identity of the cloned product was confirmed by DNA sequencing, they showed 100% similarity with the chitinase gene (GenBank Accession No. DQ873889), reported. This coding region of chitinase gene (about 1 kb) was restricted as *Bam* H I and *Xba* I fragment and gel eluted after running on agarose gel for further cloning into the pHT43 expression secretion vector. The pHT43 vector had a *lacI* repressor gene which needs induction with IPTG for the protein production to initiate. As the presence of this repressor would hinder the expression of the inserted coding region in field conditions, it is essential to remove this particular region to facilitate the continuous expression of the gene of interest. For this purpose, the region between 1266 and 1821 (555 bp) was restriction digested with *Apa* I and *Sac* I enzymes and the remaining vector was religated after filling the ends with T4 DNA polymerase. The pHT43 vector devoid of the repressor was used to transform *E.coli* cells. Deletion of this particular fragment was confirmed by restriction digestion analysis of the isolated plasmids from the transformants. Fig. 6 confirms that this vector DNA cannot be digested by *Apa* I and *Sac* I (the bands were similar to the undigested plasmid) but could be linearized with *Bam*H I (7488 bp size, similar to that of positive control, the end filled plasmid before ligation) indicating the deletion of the repressor region. Later the eluted chitinase gene was inserted into the corresponding sites of modified pHT43 vector after double digestion and purification of the vector using *Bam*H I and *Xba* I restriction enzymes and its alkaline phosphatase treatment. The appropriate clones of *E.coli*/ recombinant vector were confirmed by colony PCR (using the vector specific primers designed and synthesized during the work) and restriction digestion analysis (Fig. 7). When sequenced, the data confirmed the presence of the insert in the right direction. The recombinant vector was transformed into the *Bacillus subtilis* cells (strain WB8000N), and the transformants purified were confirmed by colony PCR and the protein was extracted from the clones. The results (Fig. 8) indicate that the protein is expressed in the medium (as extracellular protein) during the log phase without induction with IPTG. Chitinase activity (Fig. 9) when checked gradually increased from 3rd hour to 5th hour and was maintained at maximum till 7 hours of samples analyzed. This attempt has proved that the coding region of chitinase gene from *Hevea* can be successfully cloned into the *Bacillus* specific expression secretion vector and could be expressed in *Bacillus subtilis* cells. This was further transformed into the endosymbiotic *Bacillus subtilis* of *Hevea* and cofirmed by colony PCR (Fig.10). The efficiency of the standardized protocol was again found successful in both the strains. The further trials in endosymbionts are going on in this work. This attempt opens a new area in *Bacillus subtilis* research, as this recombinant vector can be used to deliver the over expressed chitinase protein or other stress related proteins from the suitable host cells of endophytic *Bacillus subtilis* into plant tissues without induction.

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