OVER-EXPRESSION OF STRESS TOLERANT GENES IN ENDO SYMBIONTS
AND ITS APPLICATION IN STRESS MANAGEMENT OF Hevea brasiliensis

Introduction

Hevea brasiliensis, the Pará rubber tree, often simply called rubber tree, is a perennial tropical plant, belonging to the family Euphorbiaceae (Archer & Audley, 1987) and the most economically important member of the genus Hevea. In India, its cultivation was started in 1870’s by the British and by the first half of the 20th century many plantations were successfully established in the country. The rubber tree is a renewable, sustainable, nonpolluting and environment friendly source of natural rubber in sharp contrast to synthetic rubber manufactured from petroleum base (Jacob, 2000). Of the ten latex bearing species reported (Wycherley, 1992), Hevea brasiliensis, the para rubber accounts for 99% of world’s natural rubber production (George and Panikkar, 2000).

The growing demand for natural rubber all over the world has emphasized the need for substantial increase in the total production of natural rubber. Productivity enhancement alone cannot bridge the gap between production and consumption and the only alternative could be expansion of area under the crop. Scope for expansion of the crop in the traditional belt is little due to non-availability of land. The only practical solution, therefore is to extend rubber cultivation to non traditional regions. Rubber is now cultivated in Tripura, Assam, Meghalaya, Nagaland, etc. in the north eastern regions, Maharashtra and Goa in the Konkan region and West Bengal, Orissa, Andhra Pradesh
and Madhya Pradesh in the eastern region. A warm humid equable climate and a well distributed annual rainfall of 2000 mm are ideal for good growth and optimum yield of *Hevea*. Rubber plants in such non-traditional areas are exposed to various abiotic stress conditions like extreme drought or cold.

Apart from various abiotic stress factors, *Hevea* also undergoes biotic stress such as wounding (caused while the trees are tapped), stimulation by ethephon (which increases the internal ethylene level), senescence before wintering and various diseases. Long term ethephon treatment also leads to a phenomenon called Tapping Panel Dryness (TPD) in bark resulting in cessation of latex flow. Most of the diseases and some of the pests that infest rubber in the traditional belts are also noted in the non-traditional areas, but the extent of damage and the order of severity vary. Among all the diseases, powdery mildew is the most severe one in the north eastern region. Wide spread occurrence of *Gleosporium* leaf disease, abnormal leaf fall caused by *Phytophthora*, *Corynespora* leaf disease, Pink disease, Brown root disease, etc. has also been reported in such regions. *Corynespora* leaf fall caused by the fungus ‘*Corynespora cassiicola’* is one of the most destructive leaf diseases affecting rubber in many Asian and African countries. It affects young and old leaves of both immature and mature rubber trees. The surveys made by the International Rubber Research Development Board (IRRDB) found this disease endemic to Indonesia, Sri Lanka and Thailand. The plant also succumbs to various leaf, stem and root diseases which are being controlled by various chemical pesticides that are again a serious threat to the eco-system because of its residual effect.

The plant produces a group of proteins called pathogenesis related proteins to alleviate such adverse conditions. Research on plant defense mechanisms has led to
special attention on chitinases, which are the first pathogen-induced proteins with its function identified. Chitinase gene has also been identified in the genome of *Hevea brasiliensis*, with its level being not sufficient enough to prevent the outbreak of fungal infections. To counter such fungal attack, it would be appropriate if the quantity of chitinase and other PR proteins are expressed at higher levels in *Hevea*.

Among the various control measures, biological control method has been found safer and to cause no side effects to the ecosystem. Biological control of plant pathogen naturally occurs at some level in all agricultural ecosystems. In the case of fungal infection, biological control is achieved by degradation of chitin, which is an insoluble, linear polymer of β-1, 4 linked NAG (N-acetylglucosamine) residues found in fungal cell wall as well as in the exoskeleton of insects and nematodes. It is the most abundant nitrogen bearing organic compound in nature. Chitinases are ubiquitous enzymes of bacteria, fungi, animals and plants which can hydrolyze the β-1,4-linkage between N-acetyl glucosamine residues of chitin.

Existence of endosymbiotic bacteria has been reported in leaf, petiole, stem, bark and in the rhizosphere of various plants such as potato (Sturz, 1995) and cucumber (Mahaffé and Kloepper, 1997). The endosymbionts residing in the rhizosphere enter the plants through the rhizoplane, epidermis and cortex (Kloepper *et al.*, 1992) and colonize. These endophytes are also reported to improve early plant growth (Nejad and Johnson, 2000), drought resistance (Sturz *et al.*, 2000, Timmusk and Wagner, 1999), atmospheric nitrogen fixing (Baldani *et al.*, 1997) and enhance the level of mineral nutrition (Bavaresco *et al.*, 2000). They have also been used for the delivery of insecticidal endotoxins (Tomasino *et al.*, 1995). These endosymbionts have also been reported to
play a major role in rendering disease resistance (Kloepper et al., 1999) as well as in enhancing the growth and development of plants (Verma et al., 2001).

Recent studies also indicate the existence of endosymbionts such as *Bacillus subtilis* in the intercellular regions of almost all parts of natural rubber trees (Philip et al., 2005). These endophytes which colonize an ecological niche similar to that of phytopathogens can be best used as bio control agents (Berg et al., 2005). It is also worth attempting to genetically modify such endosymbionts to deliver gene products that can render disease tolerance. Chitinase is one such protein that can be overexpressed in endosymbionts to prevent the plants from fungal diseases. This possibility can also be explored as an alternative to the conventional genetic transformation of the plants with the gene of interest. *Bacillus* cells that are genetically engineered to express stress tolerant proteins thus applied on the vulnerable parts of the plant before the onset of the disease or stress, may help the plants to evade from disease/stress conditions.

*Bacillus subtilis* is non pathogenic and is considered as a GRAS organism (Generally Regarded As Safe). It has no significant bias in its codon usage and it is capable of secreting functional extra cellular proteins directly into the culture medium. At present about 60% of the commercially available enzymes are produced by *Bacillus* species. A large body of information concerning its transcription, translation, protein folding, secretion mechanisms, genetic manipulation and large scale fermentation have been gathered (Harwood, 1992; Meima et al., 1995; Westers, et al., 2004; Wong, 1995). In this study, an attempt has been made to standardize an efficient transformation protocol for endosymbiotic *Bacillus subtilis* and to overexpress chitinase using a modified vector in *Bacillus subtilis* cells.
**Objective of the study**

- To standardize an efficient transformation protocol for endosymbiotic *Bacillus subtilis* cells.
- To genetically modify a recombinant vector for the constitutive expression and secretion of antifungal protein/chitinase in endosymbiotic *Bacillus subtilis* cells.
- To evaluate the level of expression of chitinase in transformed *Bacillus subtilis* cells.
- And to explore if this could be an efficient system to prevent fungal attack 

(Corynespora leaf disease) in *Hevea*.

**Materials and Methods**

A *Bacillus* specific, pHT43 expression secretion vector was purchased from Mobitec, Germany. This vector has the signal peptide that could transport the protein across cell wall. WB800N of *Bacillus subtilis* was also purchased from Mobitec, Germany which is specifically modified for the expression secretion vector pHT43. Another naturally occurring strain of *Bacillus subtilis* purchased from Genei, Bangalore (subcultured from ATCC strain 6633) was also used in the work along with the endosymbiotic *Bacillus subtilis*.

An efficient transformation protocol was standardized for endosymbiotic *Bacillus subtilis* (strain 8LK) which was also successful in WB800N and ATCC 6633 strains of *Bacillus subtilis*. The above mentioned cells were applied to poly bag plants and their colonizing capacity in the intercellular regions of *Hevea* was evaluated.

The leaves of *Hevea* plant (GT1, a disease resistant clone) were challenged with the fungus *Corynespora cassiicola* using standard procedure. mRNA was isolated by using
Dyna beads (Invitrogen, USA) and the cDNA was synthesized by using Superscript IITM RT First strand synthesis kit (Invitrogen, USA). PCR amplification of chitinase gene was performed with standard conditions using chitinase gene specific primers flanked with restriction sites (BamH I in the forward primer and Xba I in the reverse primer) to facilitate directional cloning. pGEMT Easy vector was used for cloning the PCR amplified products. Colony PCR and restriction digestion analysis were performed as per the standard protocol to select the transformed cells. DNA sequencing of the clones was carried out by Macrogen, Korea.

Further, the lacI repressor gene in the pHT43 vector restriction was removed by restriction digesting with ApaI and SacI enzymes to enable constitutive expression (in field conditions) and the modified vector was transformed into E.coli cells. Further, chitinase coding region was inserted into the modified vector followed by transformation into E.coli cells and DNA sequencing. This recombinant vector was also transformed into the mutated strain of Bacillus subtilis. The following steps were carried out to confirm the efficacy of the constructed vector in transformants. For this purpose, parameters such as PCR amplification (using gene specific primers and cDNA of its transcripts as template), chitinase activity (chitinase assay kit, Sigma) and protein visualization in SDS PAGE gel (acetone precipitation method) were performed.

The recombinant vector was further transformed into the endosymbiotic Bacillus subtilis cells (strain 8LK) and the strain 6633.

**Results**

Various protocols have been tried to evaluate the efficiency of transformation in Bacillus subtilis cells and attempts were made to evolve and standardize a better
transformation protocol. The transformation protocol using HS and LS medium was found suitable for all the three strains of *Bacillus subtilis*. The results obtained indicate that endosymbiotic *Bacillus subtilis* cells transformed after one hour in stationary phase had a better transformation efficiency than cells harvested during other periods. Its efficiency declined after an hour in stationary phase.

While the test plants were applied with *Bacillus subtilis* cells (all the strains) the control plants were treated with distilled water. Leaf samples were collected at 24 hour interval up to two weeks. The sap collected after grinding the surface sterilized leaves was spread on LB agar plates and the resultant colony forming units (cfu) were counted after overnight incubation. The result indicated that the plants treated with water, the mutated strain and strain 6633 yeilded approximately similar number of colonies whereas the plants treated with endosymbiotic strain of *Bacillus subtilis* displayed more number of colonies. While the endosymbionts of *Hevea* could recolonize the intercellular regions of *Hevea* the other two strains couldn’t colonize. This indicates indicating that only the native endosymbionts can colonize in the intercellular regions of *Hevea* and the other strains are not capable.

The leaves of the *Hevea* plants challenged with *Corynespora cassiicola* were collected after the development of symptoms. cDNA, synthesized from mRNA of the infected leaves (of *Hevea*) was used as template to PCR amplify the 978 bp coding region of chitinase gene using the gene specific primers.

The cells harbouring the pGEMT Easy vector with chitinase coding region were selected based on PCR amplification of 1kb length and by restriction digestion analysis using *Bam*HI and *Xba* I restriction enzymes. The identity of the cloned product was
confirmed by DNA sequencing, which showed 100% similarity with the chitinase gene (GenBank Accession No. DQ873889), reported (Philip et al., 2006).

The coding region of chitinase gene (about 1 kb) was restricted as Bam H1 and Xba1 fragment and gel eluted (after running on agarose gel) for further cloning into the pHT43 expression secretion vector. pHT43 vector specific primers were designed and synthesized and the annealing temperature of the primers was standardized by performing gradient PCR.

The pHT43 vector had a lacI repressor gene which needs induction with IPTG for initiating the protein production. As the presence of this repressor would hinder the expression of the inserted gene in in vivo conditions, it was necessary to remove this region to facilitate the constitutive expression. For this purpose, the region between 1266 and 1821 (555 bp) was restriction digested with ApaI and SacI enzymes and the remaining vector was religated after filling the ends with T4 DNA polymerase. The pHT43 vector devoid of the partial region of the repressor was again transformed into E.coli cells. Deletion of the repressor region was confirmed by restriction digestion analysis of the plasmid DNA isolated from the transformants. Subsequently, chitinase gene was inserted into the corresponding sites of modified pHT43 vector (recombinant vector) after restriction digesting with Bam H1 and Xba1 restriction enzymes. Colony PCR and restriction digestion of the plasmid after transforming into E.coli cells as well as DNA sequencing confirmed the presence of the insert in the right orientation.

This recombinant vector was transformed further into the Bacillus subtilis cells (strain WB8000N). The Bacillus subtilis cells (both transformed with recombinant vector and modified vector) exhibited a growth pattern different from the untransformed cells.
The *Bacillus subtilis* cells harbouring the recombinant vector reached stationary phase 1 hour later than the ones with the modified vector, which reached stationary phase 2 hours later than the untransformed cells. The rate of growth was slower in the case of transformed cells when compared to the control.

The amplification of 1kb fragment of chitinase gene from the cDNA synthesized from the total RNA indicates its expression in the transformed *Bacillus* cells. Chitinase activity gradually increased from 3\textsuperscript{rd} hour to 5\textsuperscript{th} hour which remained high till 7 hours. The chitinase protein got expressed in the liquid culture medium (as extracellular protein) during the log phase without IPTG induction. The SDS PAGE analysis also indicated the expression of approximately 34 kDa in size (with its signal peptide sequence) in the *Bacillus subtilis* transformed with the recombinant vector. This attempt has proved that the coding region of chitinase gene from *Hevea* can be successfully cloned into the *Bacillus* specific expression vector and could be expressed in *Bacillus subtilis* cells.

The recombinant vector was further transformed into the endosymbiotic *Bacillus subtilis* isolated from *Hevea*. However, the recombinant plasmid was found unstable in the endosymbiotic isolates, even within 24 hrs after transformation. The pH43 vector alone were also found not stable in the transformed endosymbionts. Growth of the transformants in the media containing antibiotic was also not possible. The *Bacillus subtilis* strain ATCC 6633 also exhibited the same trend when transformed with the recombinant vector. Modification of genome of host cell could probably help to get rid of this problem in endosymbionts. Several reports also support instability of the vector in unmodified cells.
Conclusions

The basic objective of this study was to employ the endosymbionts naturally dwelling in the intercellular regions of Hevea to express the product of gene of interest. Protocols for inducing competency and efficient transformation of endophytic Bacillus subtilis cells were standardized. The recolonizing capacity of endosymbionts (unmodified) was confirmed by leaf application experiments which make them as a promising tool for expression and secretion of protein of interest in the intercellular regions of Hevea. A modified recombinant vector was developed which can constitutively express the cloned gene in endosymbiotic Bacillus subtilis. The efficacy of the recombinant vector for expression and secretion of protein was confirmed in the Bacillus subtilis cells. But it was also found that the plasmid does not remain stable in both the endosymbionts as well as in the strain 6633 (unmodified cells). Many workers in this line have reported about structural (intermolecular recombination or intramolecular recombination) (Grandi et al., 1981, Gryczan and Dubnau, 1978, Kreft et al., 1982, Lopez et al., 1984, Ostroff and Pene 1984) or segregational instability (the loss of entire plasmid from the host) (Bron et al., 1987, Bron and Luxen. 1985, Grandi et al., 1981, Kreft et al., 1982, Rabinowitz et al., 1985). These problems often encountered in cloning experiments are independent of the general recombination systems of the host strains (Albertini et al., 1982, Farabaugh et al., 1978, Grandi et al., 1981, Ikeda et al., 1981, Jones et al., 1982, Primrose and Ehrlich. 1981, Uhlin et al., 1981). Although very high yields have been reported with homologous proteins, overproduction of heterologous proteins by B. subtilis is unfortunately highly complicated (Palva, 1982, Bolhuis et al., 1999, Olmos-Soto and Contreras-Flores, 2003, Li et al., 2004).
The structural instability due to plasmid to plasmid incompatibility or DNAse activity and segregational instability due to recombination of homologous regions can be overcome by modifications in genomic level. The WB800N, a genetically modified strain of *Bacillus subtilis*, displayed good plasmid stability and protein expression. This indicates that the endosymbiotic *Bacillus subtilis* isolated from *Hevea* also needs modifications in genomic level to make it a suitable host for overexpression studies.

The system being developed for the expression of heterologous gene does not affect the plants’ genome as there is no genomic integration. Endosymbionts can be engineered to over-express a particular gene or a group of genes by constitutive gene expression. The approach adopted in this work facilitates the constitutive expression of inserted gene. The results of this study indicate that this approach is viable in *Bacillus subtilis* cells. But the natural endosymbionts of *Hevea* need to be genetically modified to make it congenial for the colonization in the intercellular regions of *Hevea*. An additional advantage of this approach is that the foreign genes are not integrated into the genome of the bacteria. Hence there won’t be gene transfer to the wild relatives of *B. subtilis* cells in the ecosystem. This attempt opens up a new method of foreign gene insertion in *Hevea* that is devoid of to permanent insertion of gene into the *Hevea* genome. These modified cells can be applied on the leaves just before the onset of the *Corynespora* leaf fall incidence. The probably of disintegration of the plasmid after few days makes it environmentally a safer way of biocontrol. So, factors such as the relative easiness with which the cells can be transformed and multiplied, the expected higher level of expression of protein and the possibility of timely application before the onset of