A-No. 520

Journal of Plantation Crops 2006, 34(3) : 529-533

Isolation and characterization of hevein gene promoter from *Hevea brasiliensis*

A. Saleena, R. Supriya and A. Thulaseedharan*
Biotechnology Division, Rubber Research Institute of India Kottayam 686009

Abstract

Hevea brasiliensis, the Para rubber tree is the major source of commercial natural rubber and accounts for 99 % of the total world production. Hevein is an acidic chitin binding protein with high-level expression in the latex vessels of rubber tree. Wounding and application of plant hormones such as ethephon and abscisic acid were found to stimulate the hevein gene expression. In the present study, a promoter sequence of hevein gene was isolated by random amplification of genomic DNA ends (RAGE). The genomic DNA of *Hevea* clone RRII-105 was restriction digested with various enzymes. The digested fragments were ligated with specially designed adapters. The adapter ligated fragments were PCR amplified with adapter specific forward and gene specific reverse primers. Initially, with *Dra I* digested DNA a 626 bp fragment was amplified. Further, using the reverse primer based on the 5'region of the 626 bp fragment a 1416 bp second fragment was amplified. Finally, a 1934 bp fragment was amplified from the genomic DNA using the forward primer designed based on the 5' region of the 1416 bp fragment and the gene specific reverse primer. This 1934 bp fragment contains 1865 bp promoter sequence upstream to the transcription initiation codon (TIC), ATG. The isolated promoter contains 48 more nucleotides upstream to the earlier reported sequence. The promoter sequence contains the 'TATA' element at –81 and a 'CAAT' box motif at -198 position from the TIC. Several gene regulatory elements reported from other plants were also present in this sequence. Since, Hevein is a protein with high expression in latex, its promoter could be exploited for the high level expression of various recombinant proteins in the laticiferous cells of transgenic *Hevea brasiliensis*.

Key words : Hevea brasiliensis, hevein, promoter sequence, laticifer cells

Introduction

The importance of natural rubber has prompted active investigation in the molecular aspects of rubber biosynthesis in *Hevea brasiliensis*. Natural rubber, cis-1, 4-polyisoprene is obtained from the latex, the cytoplasmic content of the highly specialized cells called laticifers. Laticifers have been shown to exhibit differential gene expression and several genes involved in rubber biosynthesis and plant defense were found to have higher expression in these cells (Kush *et al.*, 1990).

Hevein is one of the major proteins present in the lutoid bodies of rubber tree latex (Archer *et al.*, 1969). This is a small single chain chitin binding protein of 43 amino acids rich in cysteine and glycine (Walujono *et al.*, 1975). Hevein is involved in wound plugging and being a chitin binding protein inhibits various chitin containing fungi (Van Parjis *et al.*, 1991). Xavier *et al.* (1993) reported the formation of polyvalent bridging between hevein and rubber particles during latex coagulation. A cDNA clone of 1018 bp encoding hevein gene was isolated earlier from a *Hevea* latex cDNA library (Broekaert *et al.*, 1990). Gene expression studies revealed a high level expression of this protein in latex. Wounding as well as the application of ethephon and abscisic acid induces the hevein gene expression in latex, leaf and stem but not in roots (Broekaert *et al.*, 1991).

Promoters are the 5' regulatory sequences responsible for the regulation of spatial and temporal expression of genes, which determines when, where and to what extent the protein, is to be expressed. Promoters contain highly

*Author for correspondence

conserved 'TATA' and 'CAAT' motifs (Joshi, 1987) and other cisacting elements, the site were the transacting regulatory proteins bind. The cisacting elements can be up regulators called enhancers or down regulators called silencers. The availability of regulatory sequences or promoters to target gene expression to appropriate cells, tissues or developmental stages is an essential component for engineering desired traits in transgenic plants. Guilley *et al.* (1982) identified the promoter element directing the synthesis of 35 S RNA in *Cauliflower mosaic* virus. Odell *et al.* (1990) identified the specific DNA sequence required for the activity of CaMV 35S promoter and also found that CaMV 35S promoter is expressed constitutively in most dicots (Rogers *et al.*, 1987). The use of fusion between promoter and reporter genes has allowed a detailed monitoring of the activity of numerous plant promoters. Promoters of hevein (Pujade- Renaud *et al.*, 2005), rubber elongation factor (AY 712939) and β-1,3glucanase (AY 325498) genes were reported earlier from *Hevea brasiliensis.*

Latex is a unique biological system and is promising for the transgenic expression of valuable recombinant proteins (Yeang *et al.*, 2002). The present work is focused on the isolation and characterization of the laticiferous specific hevein promoter and explore the possibility of using this promoter for the over expression of transgenes in the laticifer cells. Characterization of such promoters would be useful in the over expression of important genes involved in rubber biosynthesis, genes for the production of recombinant protein and also for the expression of genes induced by wounding and ethephon treatment.

Materials and Methods

The popular *Hevea* clone RRII 105 was selected for this study. Leaf samples were collected from the germplasm nursery of Rubber Research Institute of India. DNA was isolated from young leaves following the modified CTAB procedure (Doyle and Doyle, 1990). RAGE technique was followed to amplify the 5' regulatory sequence (Siebbert *et al.*, 1995). DNA was digested with blunt end cutting enzymes generate blunt-ended fragments. Several blunt end- cutting enzymes such as *Eco* RV, *Dra* I, *Ssp* I, *Stu*I, *Hpa* I, *Ass* I were tried and those which provide good digestion were selected for further study. Ten ìg DNA each was separately digested overnight at 37° C with 10 U of enzymes in 100 ìl reactions containing 10 X reaction buffer supplied by the manufacturer (New England Biolabs). *Stu* I and *Hpa* I enzymes were added at 15 ìl each. The digestion was checked in 0.8 % agarose gel. The DNA fragments were purified using the GFX™ PCR, DNA and Gel Band Purification Kit (Amersham Pharmacia, USA) following manufacturer's instruction.

An adapter was designed for this purpose, which consists of a long arm and a short arm with amino link at its 3' end to prevent any erroneous extension during PCR. Adapter Sequences are as follows:

Long arm : 5'CTAATACGACTCACTATAGGGCTC GAGC GGCCGCCCGGGCAGGT 3'

Short arm : 5' ACCTGCC C-NH_2

The two oligonucleotide adapter arms were synthesized at Sigma Genosys (India) and the adapter was prepared by mixing 100 ìM each of the two oligonucleotides and incubation at 96° C for 2 min followed by slow cooling to room temperature for annealing.

Adapter was ligated to restriction digested DNA fragments. The ligations were carried out in 20 ìl volume containing 10 ìl DNA digest, 2 ìl ligase buffer (10X), 1 ìl adapter to a final concentration of 5 ìM and 20 ligase enzyme (New England Biolabs) at 16° C and kept overnight. Several dilutions of this reaction mixture were used as PCR template using hevein gene (NCBI Accession No. M36986), specific reverse Primer I (5'TAGGGTTTATTGGGGCAGAGC3') and adapter specific forward primer I (5'ATACGACTCAC TATAGGG3'). For confirmation a nested PCR was performed with the product of first PCR as template using a second adapter specific forward primer (5'ATA GGGCTCGAGCGGC3') and gene specific reverse primer (3'TTGCCGACCACATTGCTCAG 5').

Initially a 606 bp fragment was amplified with adapter ligated *Dra* I digested DNA fragments. The amplified fragment was cloned by TOPO TA Cloning Kit (Invitrogen, USA) according to the manufacturer's instruction. Transformed bacterial colonies containing inserts were selected through blue white screening in plates containing ampicillin (50 ìg/ml), X- gal and IPTG. Recombinant plasmids were then isolated through modified alkaline lysis method (Tartof *et al.*, 1987) and sequenced (M/S Macrogen, Korea).

For the amplification of further upstream elements two reverse primers, R_1 (5'TTAACTCGATCTTATCTC3') and R_2 (5'TATCTCTATCTTATCATC3') were designed from the 5' end of previously amplified 606 bp fragment. Using these primers in combination with adapter specific forward primers, a second fragment of 1.4 Kb, upstream to the 606 bp promoter was amplified from the adapter ligated *Ssp* I DNA digests. Finally, a forward primer (5' ATTCATTAATTAATCTAT3') designed from the 5' region of the second RAGE product and gene specific

reverse primer mentioned earlier a 1964 nucleotide fragment was amplified. The PCR reaction was carried out in 20 µl volumes containing 100 µM dNTPs, 250 nM of each primer, 1.5 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Geni, Bangalore) with 20 ng template DNA in a thermal cycler (Perkin Elmer-480). The PCR conditions were: an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min and annealing at 60°C for 1 min and extension at 72°C for 2 min. The final extension was at 72 °C for 10 min. The fragment so obtained was cloned, sequenced and confirmed for the presence of promoter sequence upstream to the transcription initiation codon. The promoter sequence obtained was analyzed using the bioinformatics tool "PLACE" (Lescot *et al.*, 2002) for identification of regulatory motifs and compared with earlier reported sequences in the GenBank database.

Results and Discussion

Genome walking through adapter anchored random amplification of genomic DNA ends is one of the popular methods for the characterization of promoter elements, controlling the expression of genes. In the present study, good quality DNA was isolated from the *Hevea* leaf tissue and subjected to restriction enzyme digestion. Out of six blunt end cutting enzymes, *Eco* RV, *Dra* I, *SSp* I, *Stu* I, *Hpa* I and *Ass* I initially tried for DNA digestion, 4 enzymes, *Dra* I, *Ssp* I, *Hpa* I and *Stu* I showed uniform digestion satisfactory for RAGE.

In the initial attempt, PCR with the adapter ligated DNA fragment digested with the four enzymes using the adapter specific forward and hevein gene specific reverse primer, a 0.6 Kb region was amplified from the *Dra* I enzyme digest (Fig. 1). In the nested PCR this was again amplified as a single band and confirmed as the correct amplification (Fig. 2). The nucleotide sequencing was done after cloning the amplicon in the TOPO TA cloning vector. After the removal of adapter sequence from the 5' end and the coding sequence of the gene including the transcription initiation codon from the 3' end, a promoter sequence of 507 nucleotides was obtained. For the isolation of further upstream promoter sequence a reverse primer was designed based on the 5' end of already isolated 507 bp sequence. Using this primer and adapter based forward primer a 1.4 Kb fragment was amplified from the adapter ligated *Ssp* I DNA digested fragment, which was further confirmed by nested PCR (Fig. 3 and 4). On analysis of nucleotide sequence, this fragment contains 1358 bp upstream to the initially isolated 507 bp promoter sequence. Full length fragment with respect to second RAGE product was amplified from the genomic DNA of the clone RRII 105 using forward primer designed based on the 5' end of second RAGE product and gene specific reverse primer. Under optimum PCR conditions an approximately 2.0 Kb fragment was obtained (Fig. 5). Excluding the adapter sequence from the 5' end and gene sequence from the 3' end, a promoter region of 1865 bps was obtained upstream to the transcription initiation codon of hevein gene.

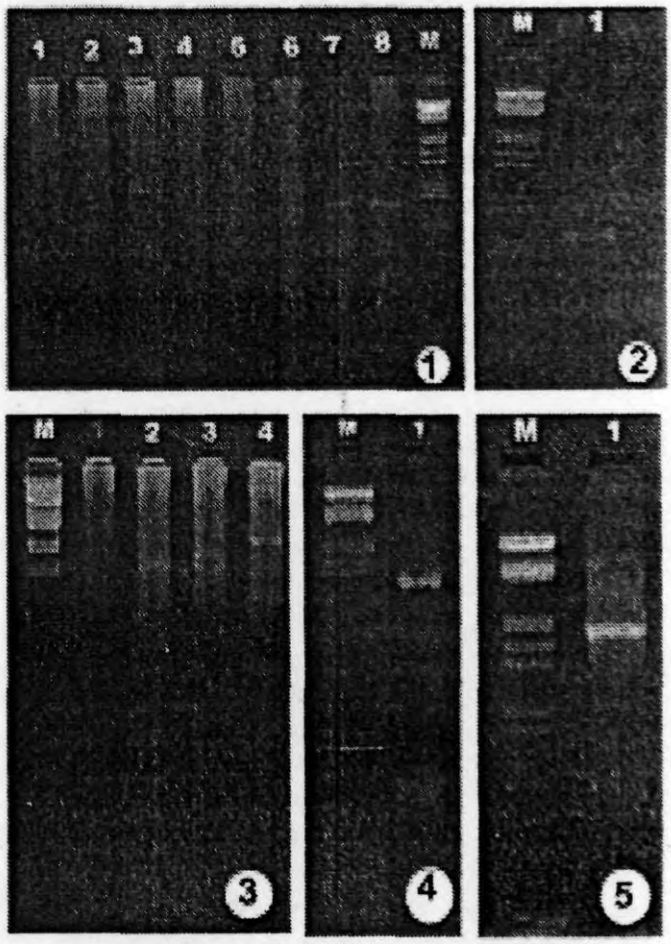

Fig. 1-5 RAGE analyses for the isolation of hevein promoter sequence

Fig. 1. I PCR (I RAGE) with adapter specific forward and gene specific reverse primer for the amplification of hevein promoter M- Mol. Wt. Marker, Lane 1-8 amplification of adapter ligated DNA digested by *SSp*I (1&2), *Hpa* (3&4), *Dra* I (5&6) and *Stu* I (7&8)

Fig. 2. Nested PCR using I PCR product as template M - Mol. Wt. Marker, Lane 1 amplification of I RAGE product

Fig. 3. I PCR (II RAGE) for the amplification of further upstream promoter sequence using reverse primer based on the 5' region of the I RAGE product M - Mol. Wt. Marker., Lane 1-4 amplification with adapter ligated *Dra*I, *Ssp* I, *Stu* & *Hpa* I digested DNA fragment

Fig. 4. Nested PCR of the II RAGE product M - Mol. Wt. Marker., Lane 1 amplification of II RAGE product

Fig. 5. Amplification of full length promoter fragment from genomic DNA M - Mol. Wt. Marker, Lane 1 Promoter sequence amplified using forward primer based on the 5' region of the II RAGE product and reverse primer based on the 5' region of hevein gene.

This hevein gene promoter sequence amplified from *Hevea brasiliensis* clone RRII-105 is AT rich and contains a putative TATA box at –81 and a CAAT box motif at –198 positions from the TIC. TATA element has a role in determining the transcription initiation site. Deletion of TATA box was found to reduce gene expression. It is reported in several systems that experimental addition or deletion of TATA box does not prevent gene expression; instead transcription initiation occurs at some other sites. CAAT box, which is found upstream to TATA box appears to have little effect in the expression of some genes, although it appears to be important for others. There are reports that insertion or excision of nucleotides between CAAT box and TATA box reduces gene expression. It is interesting to note that although hevein expression is induced by ethylene, the ethylene responsive element called GCC box, a conserved 11 base sequence ' TAAGAGCCGCC' (Ohme-Takagi *et al.*, 1995) is absent in the reported sequence. A repetition of 11 nucleotides "GAAAAATAAAT" was observed at –364 and –382 position from the TIC. From the comparison of the promoter region of the plant genes so far isolated a number of consensus motifs were reported as cisacting elements. Cisacting elements observed in this promoter includes GATA box, I box, MYB core elements, MYBST1, MAR box, WRKY elements and AMY Box.

The 1865 bp hevein gene promoter isolated in the present study from the clone RRII 105 was compared with an earlier reported 1830 bp promoter sequence by Pujade-Renaud *et al.* (2005). An additional 48 nucleotides were observed upstream to the earlier reported sequence. On comparison with the earlier reported sequence, a major deletion of 23 nucleotides "CATATTATATAT GAATAATATTT" at –1117, six nucleotide sequence deletion "CAAGGA" at –513 and addition of 9 nucleotides each at –1276 "TTGCCTCTG" and –1286 "AAAAATATT" positions from the TIC were found It has been proposed that the combination of different cis-elements and trans acting factors may produce the diversity and specificity required for the regulation of gene expression (Menkens *et al.*, 1995; Martin and Paz-Ares, 1997). The 35S CaMV promoter has been extensively used in plant biotechnology as it directs high levels of transgene expression in dicot plants. Promoter sequences that confer tissue specificity, inducibility are promising for the effective regulation of transgene expression in transgenic plants. Surprisingly, there are relatively few alternatives or adjuncts to these promoters, yet such promoters are needed for the delivery of multiple transgenic traits and help to avoid the risk of transgene silencing from the repeated use of the same promoter (Flavell, 1994; Park *et al.*, 1996). *Hevea* being a renewable source of metabolites is an ideal system for molecular farming and could be benefited from the availability of laticifer specific promoters that control the expression of transgene in target tissue.

Acknowledgements

The authors thank Dr. N. M. Mathew, Director of Research, RRII for his support and encouragement. The first and second authors acknowledge the financial support from CSIR and UGC, Govt. of India.

References

Archer, B.L., Audley, B.G.M., Sweeney, G.P. and Hong, T.C. 1969. Studies on composition of latex serum and 'bottom fraction' particles. *J. Rubber Res. Inst. Malaya* **21**: 560-569.

Broekaert, W., Lee, H.I., Kush, A., Chua, N.H. and Raikhel, M.N. 1991. Wound induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). *Proc. Natl. Acad. Sci. USA* **87**: 7633-7637.

Doyle, J. J. and Koyle, J.L. 1990. Isolation of plant DNA from fresh tissue. *Focus* **12**: 13-15.

Flavell, R.B. 1994 Inactivation of Gene Expression in Plants as a Consequence of Specific Sequence duplication. *Proc. Natl. Acad.y Sci. USA*. **91**::3490-3496.

Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. 1999. Plant Cis-acting DNA elements (PLACE) database. *Nucl. Acids Res.* **27**: 297-300.

Joshi, C.P. 1987. An inspection of the domain between putative TATA box and translation site in 79 plant genes. *Nucl. Acids Res.* **15**: 6643-6653.

Kush, A., Goyvaerts, E., Chye, M.L. and Chua, N. H. 1990. Laticifer-specific gene expression in *Hevea brasiliensis* (rubber tree). *Proc. Natl. Acad. Sci. USA* **87**: 1787-1790.

Martin, C. and Paz-Ares, J. 1997. Myb transcription factors in plants. *Trends in Genetics*. 3: 67-73.

Menkens, A.E., Schindler, U. and Cashmore, A. R. 1995. The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP protein. *Trends Biochem. Sci.* **20**: 506-510.

Odell, J.T., Nagy, F. and Chua, N.H. 1990. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* **313**: 810-812

Ohme-Takagi and Shinshi, H. 1995. Ethylene-inducible DNA binding proteins that interact with an ethylene- responsive element. *The Plant Cell* **7**: 173-182.

Park, Y.D., Papp, I., Moscone, E.A., Iglesias, V.A., Vaucheret, H. and Matzke, M. A. 1996. Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. *The Plant Journal* **9**: 183-194.

Pujade-Renaude, V., Saanier, C , Ruangsri, N., Jarunya, N. and Chrestin, H. 2005. Molecular characterization of new members of the *Hevea brasiliensis* multigene family and analysis of their promoter region. *J. Biochem. Biophys. Acta* **1127**: 151-161.

Rogers, S.G., Klee, H.J., Horsch, R.B. and Fraley, R.T. 1987. Improved vectors for plant transformation: expression cassette vectors and new selectable markers. *Meth. Enzymol.* **153**: 253-277.

Seibert, P.D., Chenchik, A., Kellogg, D.E., Lukyanov, K.A. and Lukyanov, S. A. 1995. An improved PCR method for walking in uncloned genomic DNA. *Nucl. Acids Res.* **23**: 1087-1088.

Tartof, K.D. and Hobbs, C. A. 1987. Improved media for growing plasmid and cosmid clones. *Focus* **9**: 22.

Van Parjis, J., Broekaert, W. F., Godstein, I.J. and Peumans, W.J. 1991. Hevein, an antifungal protein from rubber tree (*Hevea brasiliensis*) latex. *Planta* **183**: 258-264.

Walujono, K., Scholma, R.A., Beintema, J., Mriono, A. and Hahn A.M. 1975. Amino acid sequence of hevein. *Proceedings of the International Rubber Conference, Kaula Lumpur, Malaysia*, pp 518-531.

Xavier, G., Herve, C., Hui-Lang, T and Kush, A. 1993. Hevein a lecti like protein from *Hevea brasiliensis* (Rubber Tree) is involved i the coagulation of Latex. *J. Biological Chemistry* **269**: 9278-928

Yeang, H.Y., Arokiaraj, P., Jaafer, H., Arija, S., Arif, S., Rajamanikam S., Sharib, S., Leelavathy, R., Hamzah, S. and Logt, C. P. 200 Expression of a Functional Recombinant Antibody Fragment i the Latex of Transgenic *Hevea brasiliensis*. *J. Rubber Res.* **5**: 21 225