New insights into the novel and functional promoter sequences of β-1,3-glucanase gene from *Hevea brasiliensis*

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Abstract

β-1,3-glucanases play a major role in combating the abnormal leaf fall disease (ALF) caused by the oomycete *Phytophthora* spp. in *Hevea brasiliensis*, the major commercial source of natural rubber. In this study, partial sequences of four novel promoters of different β-1,3-glucanase genomic forms were amplified through inverse PCR from the *H. brasiliensis* clone RRII 105 and sequence characterized. This is the first report showing β-1,3-glucanase genes driven by a different set of promoter sequences in a single clone of *Hevea*. The nucleotide sequencing revealed the presence of 913, 582, 553 and 198 bp promoter regions upstream to the translation initiation codon, ATG, and contained the essential cis-elements that are usually present in biotic/abiotic stress-related plant gene promoters along with other complex regulatory regions. The amplified regions showed strong nucleosome formation potential and in two of the promoters CpG islands were observed indicating the tight regulation of gene expression by the promoters. The functional efficiency of the isolated promoter forms was validated using promoter: reporter gene (GUS) fusion binary vectors through *Agrobacterium* mediated transformation in *Hevea* callus and *tobacco*. GUS gene expression was noticed in *Hevea* callus indicating that all the promoters are functional. The transgenic tobacco plants showed no GUS gene expression. The implication of these novel promoter regions to co-ordinate the β-1,3-glucanase gene expression can be utilized for defense specific gene expression in future genetic transformation attempts in *Hevea* and in a wide variety of plant systems.

Keywords: abnormal leaf fall, *Hevea brasiliensis*, *Phytophthora*, PR-protein, systemic acquired resistance (SAR), β-1,3-glucanase

Introduction

The para rubber tree, *Hevea brasiliensis* (Wild. ex. Adr. de. Juss. Müell-Arg.), commonly known as rubber tree, a member of the family Euphorbiaceae, is the exclusive commercial source of natural rubber (NR) produced worldwide (99%), due to its good rubber yield and excellent physical properties for manufactured rubber products (Greek 1991; Asawatreratanakul et al. 2003). The present global rubber cultivation is about 1.15 million hectares, with an annual rubber production of 11 million tonnes. However, considerable yield losses occur every year when various diseases, most of which are fungal in origin, strike rubber plantations. Abnormal leaf fall (ALF) disease caused by *Phytophthora* spp. is the most economically significant fungal disease in India causing a yield loss of around 38–56% when left without fungicides for one disease season (Anu et al. 2019).
Pathogenesis-related (PR) proteins comprise a vast and heterogeneous group of proteins that are induced in plants by pathogen infection and exogenous chemicals. These proteins usually take part in the systemic acquired resistance (SAR) that develops in a resistant plant upon infection with a pathogen. SAR is a long-lasting defense signaling mechanism that usually provides broad spectrum and long-lasting resistance to secondary infections throughout the plant. During SAR, multiple defense signals are generated at the site of primary infection which subsequently triggers to protect the plant from secondary infections (Gao et al. 2014). Among the signals contributing to SAR are salicylic acid (SA) and several components of the SA pathway including the methylated derivative of SA (methyl SA, MeSA) (Park et al. 2007). Among all the induced responses, PR proteins are the most important because they can lead to increased resistance of the whole plant against a pathogen attack (Adrienne and Barbara 2006). These proteins are produced in response to pathogen attack and the promoters of the genes encoding these proteins are highly regulated by the signals produced by the pathogens. Hence these promoters become highly attractive as inducible expression systems. Therefore, promoter sequences of diverse PR protein encoding genes isolated from different plant species such as Arabidopsis, maize and poplar (Zheng et al. 2012) are important in learning the defence gene expression patterns in plants.

β-1,3-glucanases (EC. 3.2.1.39) belonging to the PR-2 family of PR proteins play an important role in both constitutive and induced defense against many pathogenic fungi (Mauch et al. 1988; Mauch and Staelhelin 1989; Jongedijk et al. 1995). β-1,3-glucanases are induced not only after pathogen infection, but also as a result of different chemical and hormonal treatments (Mauch et al. 1988; Vögeli-Lange et al. 1988). They comprise large and highly complex gene families that are not only involved in pathogen defense but also in a wide range of normal developmental processes including cell division, pollen germination, fruit ripening, seed germination, etc. The diverse physiological roles might force these enzymes to occur as multiple structural isoforms that differ in their size, iso-electric point, primary structure, cellular localization and patterns of regulation. These enzymes are thought to act directly by digesting β-1,3-glucanase in fungal cell walls especially in the oomycete fungi such as Phytophthora whose cell wall predominantly contains β-1,3-glucanase. They act by digesting fungal cell walls as well as hosting polysaccharides to produce elicitors capable of evoking hyper sensitive responses (HR) indirectly (Ebel and Scheel 1992). In distal plant tissues, local HR is often associated with the onset of systemic acquired resistance (SAR). The gain of this type of resistance is usually effective against a wide range of pathogens. It is especially associated with the transcriptional activation of a full set of marker genes, most of which encode pathogenesis-related proteins such as chitinases and 1,3-β-glucanase (Kombrink and Schmelzer 2001). Thus β-1,3-glucanase play a major role in combating the ALF disease in H. brasiliensis. An earlier study on the molecular mechanism of ALF disease tolerance in Hevea showed a differential gene expression in tolerant and susceptible clones (Thanseem et al. 2005). Although, the gene is often present in both tolerant and susceptible clones, a prolonged expression of the β-1,3-glucanase gene was observed only in the tolerant clones. The wide variation observed in the expression of β-1,3-glucanase gene suggests different degrees of transcriptional control for each step of the disease tolerance mechanism.

Despite the importance of Hevea as the source of an important industrial raw material, regulatory mechanisms of the genes involved in its disease tolerance are poorly understood. The major obstacle in precisely modifying the disease tolerance in Hevea by genetic engineering/molecular breeding is our limited knowledge on how gene expression in different steps of the defense pathway is regulated. To learn about the regulatory mechanisms behind the differential expression in Hevea and their multifunctional roles, understanding the regulatory elements residing in the β-1,3-glucanase gene promoter is highly essential. Moreover, further investigations on the disease regulation mechanism of this important tree species against a variety of fungi would be useful. In this study, we characterized β-1,3-glucanase gene promoters from the elite H. brasiliensis clone, RRII 105 which is relatively tolerant to the abnormal leaf fall disease caused by the fungus Phytophthora. The regulatory mechanisms of the isolated promoters were also investigated in Hevea and tobacco systems.

Materials and Methods

Plant material and genomic DNA isolation

For genomic DNA isolation, healthy uninfected medium mature leaves of H. brasiliensis clone, RRII 105 were collected from the trees growing in the experimental gardens of the Rubber Research Institute of India, Kottayam, India. The leaves were collected in polythene bags, placed in ice and immediately frozen in liquid nitrogen for genomic DNA isolation following the modified CTAB protocol of Doyle and Doyle (1990).
**Amplification and cloning of β-1,3-glucanase promoter**

Promoter sequences of β-1,3-glucanase gene were isolated by Inverse PCR (IPCR) as described by Ochman et al. (1988). Primers for the PCR amplification of the unknown promoter sequences were designed based on β-1,3-glucanase gene sequence (NCBI #AY325498) from H. brasiliensis reported from our lab (Thanseem et al. 2003). Restriction enzyme (RE) sites with single occurrence within the sequence (NCBI #AY325498) were identified through the Web cutter software (http://bio.lundberg.gu.se/cutter2/) and as a result Ssp I enzyme (M/S MERCK, Bangalore, India) was selected for initial digestion of DNA. Five micrograms of genomic DNA were taken in a 100 µl reaction and kept at 37°C overnight and heat inactivated at 65°C for 20 min. It was monitored for RE digestion on 1% agarose gel electrophoresis. Circularization was carried out using 2U of T4 DNA ligase enzyme (M/S New England Biolabs, England) in a 500 µl reaction containing 0.5 µg of the digested DNA and incubated at 16°C overnight. After purification, the circularized DNA was further linearized using the enzyme Bgl II (M/S New England Biolabs, England) in a 10 µl reaction volume containing 0.5 µg DNA, 1 U of enzyme and 1 µl of 10X buffer.

Primers for promoter amplification were designed from either side of the Bgl II restriction site; Forward: 5’ TAG GAA ATT CCT CTC CTT CTG 3’ and Reverse: 5’ CCT GTT ATA CCA AGG CTT GCT G 3’.

IPCR was carried out in a 20 µl reaction for 2 min at 94°C, followed by 34 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min with final extension at 72°C for 10 min and analyzed on 1.2% agarose gel electrophoresis. The DNA bands were eluted from agarose gels using the GFX™ PCR DNA and Gel band purification kit (M/S Amersham Pharmacia, USA) and cloned into the Topo TA® vector (M/S Invitrogen, USA) according to manufacturer’s instructions. Plasmids were isolated from transformed colonies using the Perfect Prep Plasmid Mini Kit (M/S Eppendorf, USA) and sequenced (M/S Macrogen, Korea).

**Bioinformatic analysis of the sequenced promoter regions**

The nucleotide sequence of various PCR products was edited to discard vector sequences flanking at either end of the insert and compared with published sequences in the NCBI database using blastn program (Altschul et al. 1990). Sequence alignment and comparison were made using the clustalW program (Thomson et al. 1994). In order to identify putative transcription factor binding sites and conserved plant cis-acting regulatory elements in the isolated promoter sequences of β-1,3-glucanase, a database analysis was performed using PLACE software (http://www.dna.affrc.go.jp/htdocs/PLACE/) (Higo et al. 1999). The nucleosome formation potential of the promoter regions was screened using RECON program (http://www.mgs.biobnet.nsc.ru/mgs/programs/recon/) (Levitsky et al. 2001). The presence of CpG islands in the promoter regions was analyzed through EMBL-EBI site (www.ebi.ac.uk/Tools/emboss/cpgplot/).

**Construction of binary vector expression cassette for functional analysis of the promoter sequences**

For the functional characterization of the β-1,3-glucanase gene promoter isolated, chimeric promoter: GUS fusion binary vectors were constructed. A total of eight binary vectors was developed, which included four constructs with full length of the different promoters (913, 582, 553 and 198 bps) obtained and the other four were deletions of the 582 and 553 bp promoters to 200 bp each (553, 582) and deletions of the 913 bp promoter with 200 and 550 bps (913, 550, 913). The full length and different deletions of the promoters were amplified from the respective Topo TA® vectors using Eco RI restriction site anchored forward and Bgl II anchored reverse primers and cloned into the pCAMBIA 1381Z TDNA vector (M/S CAMBIA vectors, Australia) upstream to the GUS gene. The primer sequences used to amplify the promoter inserts were as follows; Forward primers: 1) 5’ GGA ATT CAT TTC TTT CAT TAA AT 3’ (198 bp promoter); 2) 5’ GGA ATT GCC TAT TAA GTA TGA TG 3’ (553 bp promoter); 3) 5’ GGA ATT CAGATA TAT TCA GAT TT TCT TTT TCT TT 3’ (913 bp promoter); 4) 5’ GGA ATT CTT AGA ATT AAT ATT TT TT 3’ (913 bp promoter); 5) 5’ GGA ATT CAG ACT ATA TTT TAG 3’ (913 bp promoter deleted to 200 bp); 6) 5’ GGA ATT CCC ATG TTT AAT AGA TC 3’ (913 bp promoter deleted to 550 bp); 7) 5’ GGA ATT C AA GAG CAC TAT TAT TAT G3’ (553 bp promoter deleted to 200 bp) and 8) 5’ GGA ATT C AG ATG CAA GGA GAG T 3’ (582 bp promoter deleted to 200 bp). Reverse primers are: 1) 5’ GAA GAT CTA CCA TTA AGA AGG ATG G 3’ (198 bp promoter); 2) 5’ GAA GAT CTA CCA TTA AGA AGG ATG G 3’ (582 bp promoter deleted to 200 bp). Reverse primers are: 1) 5’ GAA GAT CTA CCA TTA AGA AGG ATG G 3’ (198 bp promoter); 2) 5’ GAA GAT CTA CCA TTA AGA AGG ATG G 3’ (582 bp promoter deleted to 200 bp). Reverse primers are: 1) 5’ GAA GAT CTA CCA TTA AGA AGG ATG G 3’ (198 bp promoter); 2) 5’ GAA GAT CTA CCA TTA AGA AGG ATG G 3’ (582 bp promoter deleted to 200 bp). Reverse primers are: 1) 5’ GAA GAT CTA CCA TTA AGA AGG ATG G 3’ (198 bp promoter); 2) 5’ GAA GAT CTA CCA TTA AGA AGG ATG G 3’ (582 bp promoter deleted to 200 bp).
precipitation of DNA using 2V ethanol. Vectors were dephosphorylated using 0.5 U of calf intestinal alkaline phosphatase (CIP) (M/S New England Biolabs, England) and purified by phenol: chloroform (1 : 1) extraction. The dephosphorylated vector and the purified insert DNA (1 : 10) was ligated using T₄ DNA ligase (M/S New England Biolabs, USA) at 16°C overnight. Two µl each of the ligation mixtures were used to transform chemically competent Escherichia coli DH5α cells following heat shock for amplification of the binary vector. Recombinant plasmids were isolated and sequenced to confirm the presence and orientation of the inserts.

The competent cells of Agrobacterium tumefaciens strain EHA 105 were prepared following the method reported by Jyothishwaran et al. (2007). Agrobacterium competent cells were transformed by freeze-thaw (Holsters et al. 1978). A negative control was also constructed by transforming A. tumefaciens with original pCAMBIA 1381 T-DNA vector having GUS gene without promoter. The screening of the putatively transformed colonies of Agrobacterium was done through colony PCR using the insert specific primers.

Plant material and growth conditions

Hevea calli and leaf discs from the regenerated tobacco shoots were directly used for Agrobacterium mediated genetic transformation. Hevea calli were induced from leaf explants collected from glass house grown plants of H. brasiliensis clone RRII 105 (Kala et al. 2006). Sterile tobacco (Nicotiana tabacum L. Petite Havana strain) plants were developed from leaves obtained from glass house-grown plants. Surface sterilized leaves were grown on MS medium supplemented with 1.0 mg · l⁻¹ BAP and 0.10 mg · l⁻¹ NAA. After 2 weeks, shoots emerged directly from the explants.

Genetic transformation of Hevea and tobacco

The transformed A. tumefaciens were grown in shaking liquid LB medium at 28°C till OD₆₆₀ was 1.3. The culture was resuspended in MS (Murashige and Skoog 1962) liquid medium. Fifty µl of the re-suspended culture was added to 5 ml MGL medium (tryptone – 5 g · l⁻¹, yeast extract – 2.5 g · l⁻¹, NaCl – 5 g · l⁻¹, glutamic acid – 1.16 g · l⁻¹, KH₂PO₄ – 250 mg · l⁻¹, MgSO₄ · 7H₂O – 100 mg, biotin 1.0 mg · l⁻¹; pH 7.0) containing 20 µg · ml⁻¹ rifampacin and 50 mg · l⁻¹ kanamycin (Jones et al. 2005). Two and a half ml of the culture, incubated overnight at 28°C at 250 rpm was transferred to 7.5 ml of TY medium (pH 5.5) (tryptone – 5 g · l⁻¹, yeast extract – 3 g · l⁻¹) containing the antibiotics and 200 µM acetylsyringone. The cultures were incubated overnight at 28°C at 250 rpm. The following day 1.5 ml of the bacterial culture was diluted to 20 ml by adding TY medium (pH 5.5), containing 40 mg · l⁻¹ acetylsyringone. The OD was measured against TY blank at 600 nm and adjusted to the optimum level (0.1–0.2 OD) for transformation.

Sterile tobacco leaf discs and Hevea leaf calli were vacuum infiltrated (40 psi for 10 min) with respective Agrobacterium cultures, co-cultivated for 2 days in the co-cultivation medium (MS supplemented with 1.0 mg · l⁻¹ BAP and 0.10 mg · l⁻¹ NAA) and kept in dark. After 2 days the explants were rinsed with distilled water and checked for transient expression of GUS gene via transient assay by placing in a solution of X-gluc and incubated at 37°C overnight (Jefferson et al. 1987). Leaf discs were washed with sterile water thrice to remove excess bacterial residue, blotted dry and transferred to regeneration medium (MS supplemented with 1.0 mg · l⁻¹ BAP, 0.1 mg · l⁻¹ NAA, 30 mg · l⁻¹ hygromycin and 300 mg · l⁻¹ cefotaxime) in 16 h day length at 22°C until shoots regenerated. The regenerated transgenic shoots were transferred to MS medium devoid of any growth regulators. Insertion of promoter: GUS fusions were confirmed through respective PCRs (GUS: Forward primer 5’ TAG AGA TAA CCT TCA CCC GG 3’, Reverse primer 5’ CGC GAA AAC TGT GGA ATT GA 3’). Induction with 0.1% salicylic acid (Ding et al. 2002) was carried out in the regenerated tobacco leaves. GUS expression in the transgenic leaves was monitored by histochemical staining of GUS activity. The chlorophyll content of leaf tissues was removed by washing with 70% alcohol (v/v).

Results and Discussion

Isolation and sequence analysis of β-1,3-glucanase promoters from Hevea brasiliensis

The inverse PCR method with SpI restricted genomic DNA (Fig. 1A) was employed to isolate different β-1,3-glucanase promoters present in H. brasiliensis. Agarose gel electrophoresis of the PCR reactions revealed the presence of four distinct amplicons of approximately 1.2, 0.8, 0.76 and 0.4 kb (Fig. 1B). The four amplicons obtained through inverse PCR, upon analysis showed that the isolated regions belonged to the β-1,3-glucanase gene in Hevea. A single inverse PCR reaction yielded four amplicons indicating the difference in positions of restriction enzyme sites in the isolated genomic DNA, providing evidence that they belong to different β-1,3-glucanase genes. Efficient cloning and sequencing of the four amplicons, followed by a comparison of isolated sequences with an
earlier reported β-1,3-glucanase gene sequence (NCBI #U22147; #A325498), showed the presence of novel sequences flanked on 5’ and 3’ ends with known nucleotide regions. The promoter sequences were thus identified after eliminating the cloning vector sequences and known coding regions. The 1.2 kb amplicons contained a total of 1126 bps, which comprised 913 bp of the promoter region, while the remaining nucleotides were known coding regions. The 0.8 kb amplicon contained a 582 bp promoter sequence. From the 0.76 kb amplicons, a 553 bp promoter sequence was isolated, while from the 0.4 kb amplicon, a 198 bp promoter region was isolated.

Sequence analysis of the isolated β-1,3-glucanase promoter sequences

The BLASTn analysis of the obtained promoter sequences showed 97–100% similarity in the 5’UTR region, with the reported gene sequences of β-1,3-glucanase from *H. brasiliensis*. The coding region downstream to the newly obtained regulatory region of different promoter forms also aligned to the reported sequences of β-1,3-glucanase gene in the GenBank with minor nucleotide variations, confirming that all the promoter sequences amplified in the present study belonged to the β-1,3-glucanase gene itself. The nucleotide sequence comparison of the four β-1,3-glucanase gene promoter forms was made through clustalW (Fig. 2). Upon alignment of the four different promoters, it was observed that the promoter regions at the upstream distal end from the ‘ATG’ showed many nucleotide variations whereas the nucleotides towards the 5’UTR were 70% homologous with minor nucleotide variations in the number of nucleotides. The known regions showed significant alignment in the coding and 5’UTR regions among the four sequences that represent multiple forms of promoters that may regulate four different forms of the β-1,3-glucanase gene. When each of the sequences was considered, the 582 bp sequence showed more homology towards the sequence belonging to the 198 bp promoter (score = 71). The sequence with the 913 bp promoter showed more homology towards the sequence than with the 553 bp promoter (score 74).

The *cis*-elements in the isolated promoter sequences, recognized through PLACE software, contained essential regulatory elements that are usually present in a biotic/abiotic stress related gene promoter. They contained the TATA, CAAT, GATA, WRKY, W box, Myb elements along with other complex regulatory regions. The significance of the *cis*-elements and their frequency of occurrence in the four isolated regions of glucanase gene promoter are presented in Table 1. The promoter regions identified were rich in ‘AT’ base pairs similar to most other gene promoters.

Sequence analysis showed the presence of essential *cis*-elements providing additional evidence that the isolated sequences were promoters. They contained core promoter elements, TATA boxes and CAAT boxes positioned in relation to translation initiation codon. The proximal region of β-1,3-glucanase promoter contained the elements shown to be necessary for an accurate initiation of basal transcription in promoters. Highly conserved *cis*-element sequences identified in the promoters of special classes of plant genes, were present in the isolated promoter region (Table 1), such as, W box and WRKY sequences in pathogen responsive genes, 1 box in light inducible gene promoters and GT1 box in promoters of genes responsive to stress (Jaiswal *et al.* 2007). This reflects the fact that *Hevea* has developed a unique system to counteract various biotic/abiotic stresses. WRKY homologs constitute a large family of DNA-binding proteins in plants that are involved in several key cellular functions, including disease resistance, stress response, dormancy and development (Eulgem *et al.* 2000; Johnson *et al.* 2002; Pan *et al.* 2009). These factors act by directing the temporal and spatial expression of specific genes, thereby ensuring proper cellular response to internal and external stimuli (Ulker and Somssich 2004). Several defense related genes in plants have multiple copies of W-boxes in their promoters that are recognized by WRKY proteins and are necessary for the inducible expression of these genes (Kawagoe and Murai 1996; Droge-Laser *et al.* 1997).

*Myb* core, *Myb ST1*, *Myb consensus* and DOF core domains were also identified in the isolated
Fig. 2. ClustalW multiple sequence alignment of the four promoter fragments obtained through inverse PCR. Promoter sequence (capital font), gene sequence (small font), initiation codon ATG (bold, capital font), exon sequence (capital font, underlined)
Table 1. The putative cis-elements and their frequencies of occurrence in the isolated promoter regions of different forms of β-1,3-glucanase from the clone RRRI 105

<table>
<thead>
<tr>
<th>Cis-element/consensus</th>
<th>Function/Response</th>
<th>β-1,3-glucanase gene promoter</th>
<th>Consensus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>913 bp</td>
<td>582 bp</td>
</tr>
<tr>
<td>Amy Box 1</td>
<td>amylase box—responsible for gibberellic acid induced expression</td>
<td>1 1</td>
<td>1</td>
</tr>
<tr>
<td>CAAT Box</td>
<td>site for RNA transcription factor</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>DOF core</td>
<td>signal responsive and/or tissue specific gene expression</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>E Box</td>
<td>plant pathogen interaction</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>GATA Box</td>
<td>light responsive element</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>GT1 consensus</td>
<td>cell type specific</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>I Box core</td>
<td>light regulated and tissue specific expression</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Myb core</td>
<td>regulatory roles in developmental processes and defense responses in plants</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Myb consensus</td>
<td>regulatory roles defense responses and dehydration stress in plants</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Myb ST1</td>
<td>dehydration stress</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Myc consensus</td>
<td>dehydration induction</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Root Motiff</td>
<td>expression in root</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>TATA box</td>
<td>directing RNA Pol II to the initiation site</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Wbox(TGACY)</td>
<td>stress/pathogen defense regulated expression</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>(Zn finger)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WRKY</td>
<td>stress/pathogen induced expression</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Hevea β-1,3-glucanase gene promoter. Myb proteins are known to play important roles in various developmental processes such as controlling secondary metabolism, regulating cellular morphogenesis and serving in the signal transduction pathways responding to plant growth regulators. The plant Myb elements also regulate the expression of PR proteins (Jin and Martin 2000). DOF core domains act as transcription activation domains in vivo (Yanagisawa 1997). The variation in the type and frequency of occurrence of the regulatory elements in the isolated promoter fragment of β-1,3-glucanase gene can cause a difference in their expression patterns towards various biotic/abiotic stresses. It was also noted that the ‘IAAGAGCCG GCC’ motifs (GCC-boxes) which are commonly present in most of the pathogen inducible promoters, were absent in these promoter sequences.

Analysis of the nucleosome formation potential and CpG islands in the isolated promoter regions

Nucleosomes are the major structural elements of a chromatin and each nucleosome is formed by a DNA fragment with a length of 147 bp wrapped around an 8-mer comprising pairs of four types of histones (Luger et al. 1997). It has been reported that nucleosomes at the promoters of genes regulate the accessibility of the transcription machinery to DNA. It thus functions as a basic layer in the complex regulation of gene expression (Rudnizky et al. 2017). Understanding the precise locations of nucleosomes in a genome is the key to understanding how genes are regulated (Jiang and Pugh 2009). The profiles of nucleosome formation potential identified through the RECON program showed the probability of nucleosome formation along the promoter region amplified. Within the 913 bp promoter region, a very strong nucleosome formation potential was found in the region between nucleotides 85–220, 399–486 and 626–834 nucleotides with value ranges between 0.5–0.9. In the 582 bp promoter, maximum nucleosome formation potential was recognized within a nucleotide range of 131–165, 225–268, 294–412 bps. In the 553 bp promoter, regions with high potential for nucleosome formation were within the regions 81–144 bps and 163–474 bps. In the 198 bp, the nucleosome formation potential was found at the region 81–119 nucleotides. All positions
of the nucleotides mentioned were in the 5’-3’ direction. The isolated promoter regions of β-1,3-glucanase gene showed high nucleosome formation potential in some regions, typical of the promoter sequences as they exhibit intermittent high and low nucleosome forming tendencies. It was found earlier that in promoters of tissue-specific genes, the nucleosome formation potential was essentially higher than with the genes expressed in other tissues, or the housekeeping genes. Hence, nucleosome positioning participates in gene regulation since the DNA packing on the surface of the histone octamer can occlude the binding sites of transcription factors (TFs) on genomic DNA. Thus, the nucleosome positioning at promoters negatively regulates gene transcription events (Liu et al. 2018). The nucleosome positioning controls both the nucleosome packaging in discrete chromatin regions and the accessibility of transcription factor binding sites (TFBS) (Kiyama and Trifonov 2002). Thus, gene transcription is usually dependent on cis-element positioning and chromatin structure.

CpG islands are essential for the fine-tuned regulatory processes of the cell by regulating gene expression patterns and cell fate, thereby acting as vital landmarks of the epigenome (Jung and Pfeifer 2013). EMBOSS CpG software identified two CpG islands in the 913 bp promoter, first in the region 449–521 and second in the region 609–695 nucleotides (Fig. 3A). No CpG islands were observed in the 582 bp promoter. In the 553 bp promoter, the CpG island search showed the presence of a single CpG island (Fig. 3B). In the 198 bp promoter also no CpG island was observed, probably, the CpG islands may be present upstream to the amplified 198 bps. The two CpG islands detected in the 913 bp and one in the 553 bp promoter regions may aid in the continuous or tightly regulated expression of the gene. Some of the plant promoters similar to that of the animal promoters were found to be associated with CpG islands (Antequera and Bird 1993) and have certain relationships with gene regulation through DNA-methylation. Detection of regions of genomic sequences that are rich in the CpG pattern is important because such regions are resistant to methylation and tend to be associated with genes which are frequently switched on. Unlike CpG sites in the coding region of a gene, in most instances, the CpG islands of promoters are un-methylated if genes are expressed (Feil and Berger 2007). The presence of nucleosomes and CpG islands in the promoter region may be inter-related in the regulation of glucanase genes in Hevea.

Functional analysis of the promoter

In order to understand if all the β-1,3-glucanase gene promoter forms identified in the present study are functional, promoter: GUS fusion binary vectors were generated and the efficiency of these promoters to drive GUS gene expression was monitored in Hevea and tobacco.

The transient assay using Hevea callus showed relatively good GUS activity with all the constructs made, from the least minimum base pairs (198 bp) to the highest (913 bp) (Fig. 4). PCR of the recombinant plasmids and transformed E. coli cells also confirmed the presence of the marker gene hpt II and the GUS reporter gene. The control callus samples transformed with the promoter-less binary vector, non-transformed callus and the Agrobacterium broth culture did not show any signs of GUS activity which clearly indicated that the GUS expression obtained in the transformed calli was due to the activity of the promoter inserted.

The GUS gene expression in the Hevea callus driven by the β-1,3-glucanase gene promoters might be due to the identical tissue homogeneity and the presence of the cis-acting protein factors in Hevea genome driving the regulation of these promoters. Being the native system, Hevea callus might provide the required transcription factors (trans-acting proteins). Original glucanase gene expression was in Hevea callus, so it must have all the required factors. Expression of the GUS reporter gene in the other two constructs and their deletion constructs showed more or less a very similar pattern of regulation in Hevea callus. The results

Fig. 3. CpG island prediction using the EMBL – EBI-EMBOSS programme. A – CpG island present in the 913 bp promoter (number of CpG islands – 2; 449..521, 609..695); B – CpG island present in the 553 bp promoter (number of CpG islands – 1; 445..497)
When the GUS activity of these transgenic shootlets was checked, they did not show any GUS activity. This result is in agreement with the absence of GUS gene expression in the initial transient assay. When the emerged transgenic shoots were induced with 0.1% salicylic acid they showed a slight color change. This seems to be due to the GUS activity which was observed initially whereas, upon treatment with alcohol the coloration was not retained, indicating lack of noticeable GUS activity (Fig. 7). The negative controls (non-transformed leaf discs, leaf discs transformed with promoter-less construct) did not show any signs of GUS activity.

In the mature transformed tobacco leaves no GUS gene activity was observed when tested with and without induction with 0.1% salicylic acid. The failure of GUS gene expression in tobacco leaves may be due to the absence of enhancer elements (residing in the upstream region of the promoter)/trans elements in tobacco or due to the inability to compete for a limiting amount of its cognate transcription factor(s) or related proteins. Strong transcriptional enhancer sequences can activate gene expression in the vicinity of the site where the enhancer is inserted into the genome (Chalfun-Junior et al. 2006). In tobacco, a region has been identified from –1452 to –1193 in a glucanase gene promoter containing two copies of the heptanucleotide ‘AGCCGCC’ , which is also highly conserved in plant-stress and defense-related genes, and it is necessary for high level expression in leaves (Vögeli-Lange et al. 1994). This region was absent in the isolated region of all the promoter forms including the one with 913 bp.

**Ectopic gene expression and the effect of salicylic acid in transgenic tobacco**

The transient assay of the transformed tobacco leaf discs with all of the eight constructs did not show any detectable levels of GUS activity. After 10–15 days, transformed shoots were emerged from the putatively transformed leaf discs in the selection medium (Fig. 5A–E) whereas the non-transformed tissue gradually became bleached and finally turned dark (Fig. 5F). The hygromycin (30 mg -1) used was found to be effective for the selection of transformed tobacco. Integration of DNA constructs with respective promoter: GUS fusions in transformed plants were confirmed through PCR amplification with respective promoters for the reporter GUS (~700 bp) and marker hpt II (~610 bp) genes (Fig. 6).

![Fig. 4. Transient GUS gene expression in Hevea callus with the binary vectors carrying different β-1,3-glucanase gene promoter forms. A – GUS expression by the four isolated promoter forms; B – GUS expression by three promoter forms having nucleotide deletions; C: a) callus transformed with promoter-less binary construct, b) non-transformed callus, c) EHA 105 culture transformed with 913 bp promoter construct](image_url)
promoter. Probably the region may be present with the further upstream regions of the promoter sequences identified. This may also be a reason for the absence of GUS gene expression driven by the glucanase gene promoters in the transgenic tobacco leaves or due to the less sufficient upstream sequence necessary for the multiple cis-regulatory elements to drive the expression. All promoters need not drive genes in heterologous systems, which depend on how specific they are. It also depends on whether the required transcription factors can be met from the system. Qi-La et al. (2008) reported a very low GUS activity in the leaves of *Nicotiana* when transformed with the 1157 bp promoter of a *Gastrodia* antifungal protein.

**Fig. 5.** Regeneration of transformed tobacco plantlets in the selection plate. A – tobacco leaf discs transformed with promoter-less construct; B – tobacco leaf discs transformed with 913 bp promoter constructs; C – tobacco leaf discs transformed with 582 bp promoter constructs; D – tobacco leaf discs transformed with 553 bp promoter constructs; E – tobacco leaf discs transformed with 198 bp promoter constructs; F – non-transformed leaf discs (negative control)

**Fig. 6.** PCR amplification for confirmation of transformation in transgenic tobacco plants. A – PCR amplification of inserted promoter regions (M – λ DNA EcoRI/HindIII double digest marker; 1 – 198 bp, 2 – 553 bp, 3 – 553, 4 – 582 bp, 5 – 582, 6 – 913 bp, 7 – 913 bp, 8 – 913 bp); B – amplification of GUS gene amplification from transformed tobacco with the 8 binary vector constructs (M – λ DNA EcoRI/HindIII double digest marker, 1–8 GUS gene amplified from samples in the order as in A above); C – amplification of hptII gene from transformed tobacco with the 8 constructs (M – λ DNA EcoRI/HindIII double digest marker), 1–8: hptII gene amplified from samples in the order as in A

**Conclusions**

The present work is the first report on the occurrence of multiple promoter sequences of the β-1,3-glucanase gene in a single clone of *H. brasiliensis*. The isolated promoter sequences of the β-1,3-glucanase gene contained essential cis-elements usually present in a pathogenesis related promoter conferring high nucleosome forming potential. The occurrence of different promoter sequences itself proves the differential/tissue specific regulation of the gene as β-1,3-glucanase genes perform different actions in different tissues varying from bud formation to plant defense.
also provides new insights into the specific regulatory properties of glucanase gene promoter. In plants like *H. brasiliensis*, tissue specific and inducible promoters are very important for the development of transgenic plants for enhanced rubber production which occurs in specific tissues. For the enhanced defense mechanism, defense gene specific promoters are also very essential. Results of this work demonstrated that the partial β-1,3-glucanase promoter sequences of *H. brasiliensis*, in fact, will be able to resolve this issue as it can be used to drive the expression of genes for tolerating disease conditions. However, it would be more promising if we could isolate further upstream promoter sequences and their functional characterization. The cloned gene promoter sequences would be most useful for future investigations on the disease regulation of *H. brasiliensis* against a variety of fungi, as it could ensure maximum expression along with favorable features of nucleosome forming potential and CpG regulation of gene expression. Nevertheless, accurate recognition of the promoter structure relies on a comprehensive list of elements within the non-identified regions. The study also provides evidence for the tissue-specific expression of β-1,3-glucanase gene promoter signifying the importance of using these promoters to drive the expression of genes that can combat biotic stress.

**Fig. 7.** Transgenic tobacco leaf bits showing no GUS activity after transforming with promoter: GUS fusion binary vectors. Tobacco leaf transformed with the binary vector carrying intact promoters and their 200 and 550 bp deletion constructs along with controls – salicylic acid induced and non-transformed; salicylic acid non-induced non-transformed (negative control); transformed with construct without promoter (negative control)
References


Anu K., Limiya J., BinduRoy C. 2019. An insight into Hevea transformation systems. The control of ALF disease in Hevea plants by transferring disease resistance genes using suitable and efficient promoters can increase the yield of natural rubber. In the future, studies can be extended to identify additional sequences upstream to the isolated region of these promoters to understand whether these promoter sequences are inducible or not by physical/biological stresses when introduced in plant systems other than Hevea.


in Hevea transformation systems. The control of ALF disease in Hevea plants by transferring disease resistance genes using suitable and efficient promoters can increase the yield of natural rubber. In the future, studies can be extended to identify additional sequences upstream to the isolated region of these promoters to understand whether these promoter sequences are inducible or not by physical/biological stresses when introduced in plant systems other than Hevea.