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ORIGINAL ARTICLE

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 11.5 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 Staehelin 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 T_4 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 10 X buffer.

Primers for promoter amplification were designed from either side of the *Bgl* II restriction site; Forward: 5' TAG GAA ATT CCT ACC 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 in TopoTA® 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 done with 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.bionet.nsc.ru/mgs/programs/recon/) (Levitsky *et al.* 2001). The presence of CpG islands in the promoter regions was analyzed through EMBOSS CpG at the 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_{200}, 582_{200}) and deletions of the 913 bp promoter with 200 and 550 bps (913_{550}, 913_{200}). 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 TTC CAT TAA AT 3' (198 bp promoter); 2) 5' GGA ATT CGC TAT TAA GTA TGA TG 3' (553 bp promoter); 3) 5' GGA ATT CAG ATA TAT TCA GTT TC 3' (582 bp promoter); 4) 5' GGA ATT CTT AGA ATT AAA ATT TT 3' (913 bp promoter); 5) 5' GGA ATT CAG AGC ACT ATA TTT TAG 3' (913 bp promoter deleted to 200 bp); 6) 5' GGA ATT CCA TCC ATG TTT AAT AGA TC 3' (913 bp promoter deleted to 550 bp); 7) 5' GGA ATT C AA GAG CAC TAT ATT 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' (582/553 bp promoters) and 3) 5' GAA GAT CTA CCA TTA AGA AGG CGG G 3' (913 bp promoter). (The restriction site is underlined).

PCR amplified promoter fragments from the cloned TOPO TA® and the binary TDNA vector, pCAMBIA 1381 Z (1.0 µg each) were digested with Eco RI (M/S Bangalore Genei, Bangalore) followed by heat inactivation of the enzyme at 65°C for 20 min and a second digestion with the enzyme Bgl II (M/S New England Biolabs, UK). Digested samples were purified by phenol: chloroform (1 : 1) extraction followed by

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 T_4 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 tabaccum* 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 OD_{600} 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}, KH_2PO_4 – 250 mg · l^{-1}, $MgSO_4 \cdot 7H_20$ – 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 acetosyringone. 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} acetosyringone. 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' and hygromycin gene: Forward primer 5' CGA TTG CGT CGC ATC GAC 3', Reverse primer: 5' CGT GCA CAG GGT GTC ACG 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 *Ssp* I 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

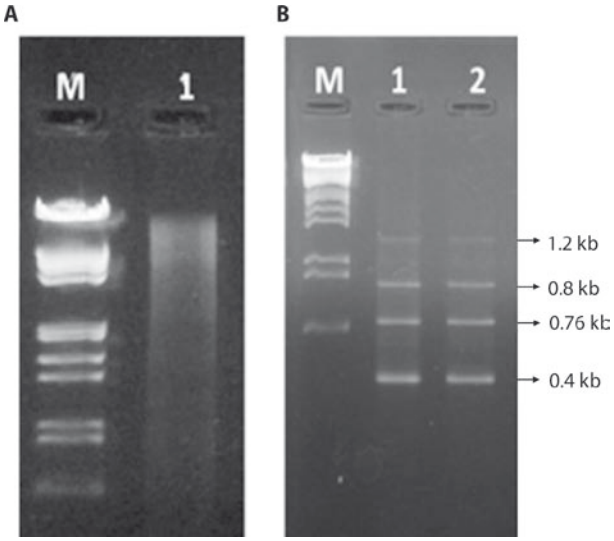

Fig. 1. Isolation of β-1,3-glucanase promoter regions. A – restriction digestion of genomic DNA with *Ssp* I enzyme: M – marker (*Eco*R1/HindIII double digest λ DNA), 1– *Ssp* I digested DNA; B – inverse PCR amplification of promoter regions of β-1,3-glucanase gene: M – marker (*Eco*R1/*Hind*III double digest λ DNA), 1 and 2 – PCR amplicons

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 genes 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, I 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

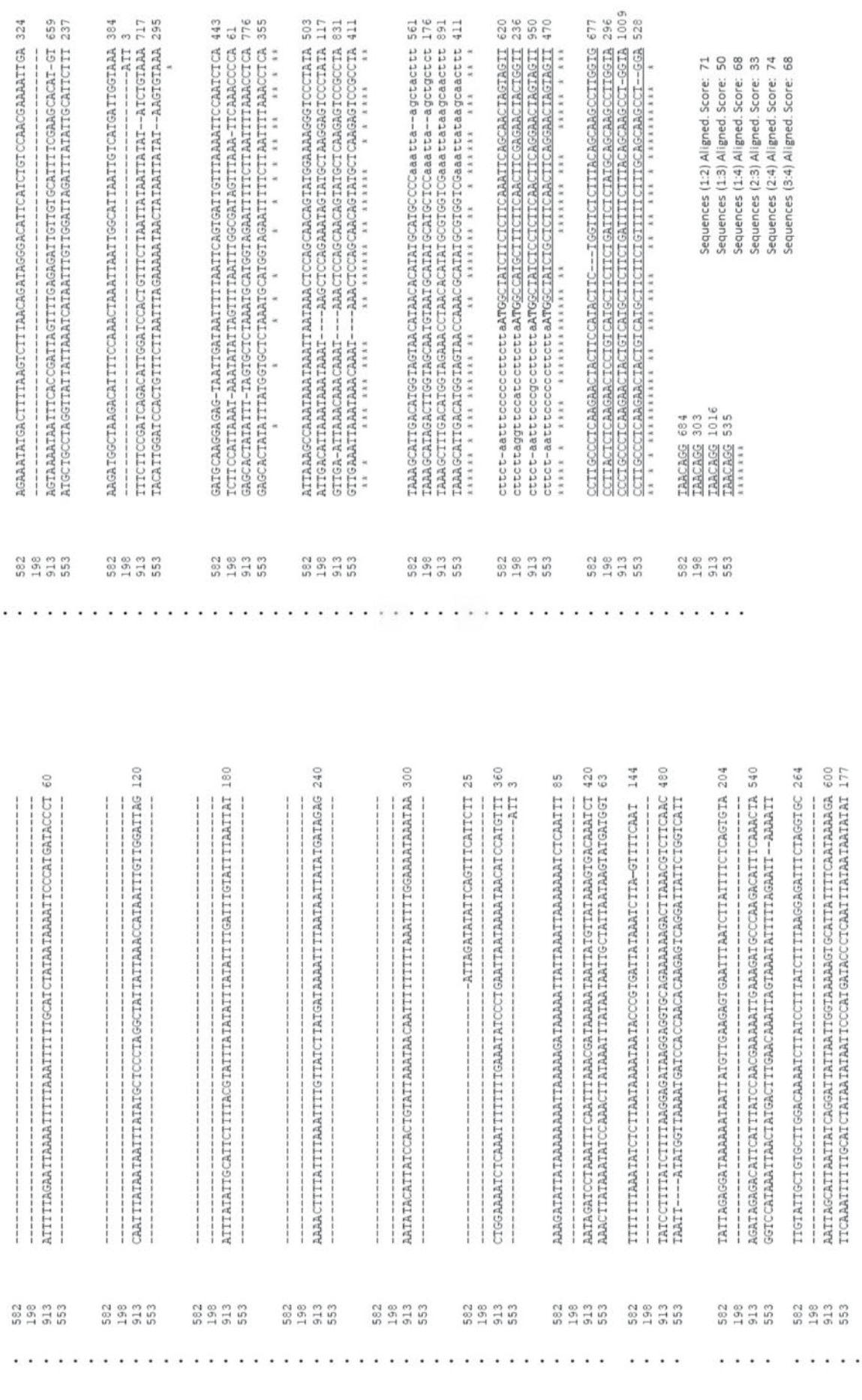

Fig. 2. ClustalW multiple sequence alignment of the four promoter fragments obtained through inverse PCR. Promoter sequence (capital font), gene sequence (small font), initiation codon ATG (bold, capital font), exon sequence (capital font, underlined)

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 RRII 105

Cis-element/ consensus	Function/Response	β-1,3-glucanase gene promoter				Consensus sequence
		913 bp	582 bp	553 bp	198 bp	
Amy Box 1	amylase box-responsible for gibberellic acid induced expression	1	1	–	–	TAACARA
CAAT Box	site for RNA transcription factor	9	13	6	3	CAAT
DOF core	signal responsive and/or tissue specific gene expression	13	12	5	1	AAAG
E Box	plant pathogen interaction	6	4	4	4	CANNTG
GATA Box	light responsive element	13	9	2	1	GATA
GT1 consensus	cell type specific	17	11	4	–	GRWAAW
I Box core	light regulated and tissue specific expression	13	7	–	–	GATAAGR
Myb core	regulatory roles in developmental processes and defense responses in plants	4	2	2	–	CNGTTR
Myb consensus	regulatory roles defense responses and dehydration stress in plants	2	-	1	–	YAACKG
Myb ST1	dehydration stress	4	2	1	–	GGATA
Myc consensus	dehydration induction	6	4	4	4	CANNTG
Root Motiff	expression in root	6	4	7	2	ATATT
TATA box	directing RNA Pol II to the initiation site	9	8	12	5	TATAA
Wbox(TGACY) associated with WRKY (Zn finger)	stress/pathogen defense regulated expression	1	3	3	1	TTGAC
WRKY	stress/pathogen induced expression	2	3	4	1	TGAC

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 'TAAGAGCCGCC' 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*.

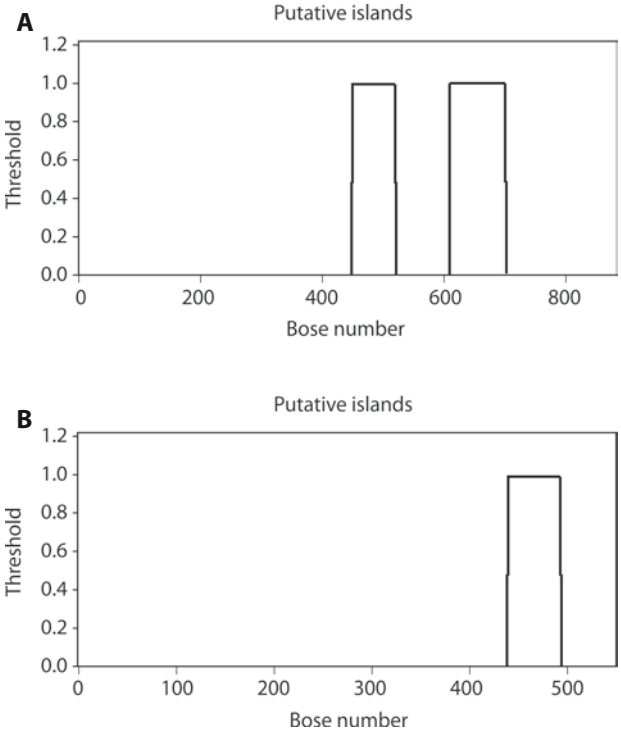

Fig. 3. CpG island prediction using the EMBL – EBI-EMBOSS programme. A – CpG island present in the 913 bp promoter (number of CpG islands – 2; 449..521, 609..695); B – CpG island present in the 553 bp promoter (number of CpG islands – 1; 445..497)

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

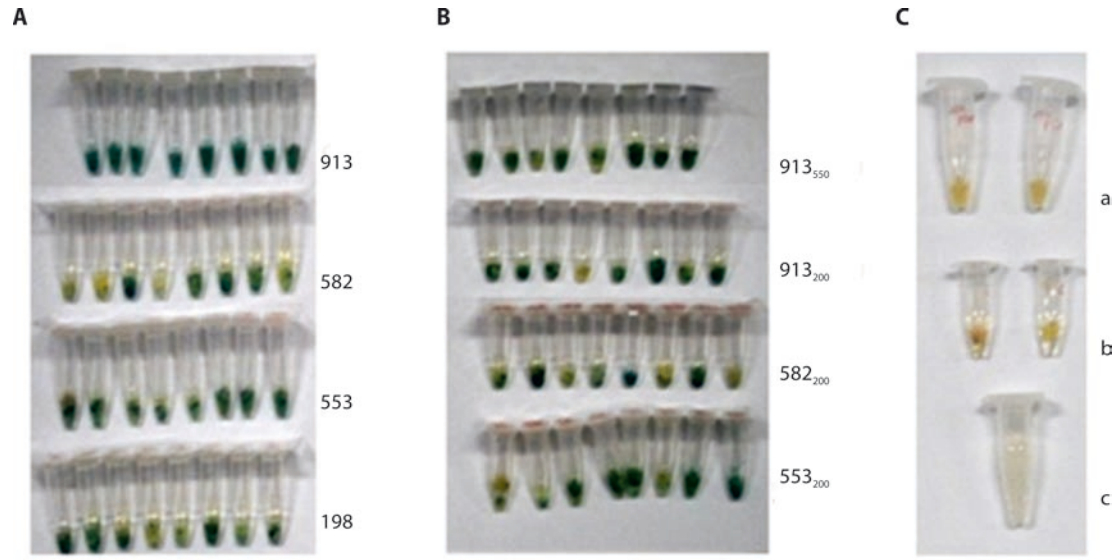

Fig. 4. Transient GUS gene expression in *Hevea* callus with the binary vectors carrying different β-1,3-glucanase gene promoter forms. A – GUS expression by the four isolated promoter forms; B – GUS expression by three promoter forms having nucleotide deletions; C: a) callus transformed with promoter-less binary construct, b) non-transformed callus, c) EHA 105 culture transformed with 913 bp promoter construct

of deletion analysis suggest that multiple positive and negative elements in the glucanase gene promoter regulate its activity. The experiment further demonstrates that the trans-activation profile for these isolated regions and their deletion constructs was specific. Unlike constitutive expression of genes, tissue-specific expression is the result of several interacting levels of gene regulation. As such, it is preferable to use promoters from homologous or closely related plant species to achieve efficient and reliable expression of transgenes in particular tissues. This is one of the main uses for the β-1,3-glucanase gene promoter from *H. brasiliensis*. *Agrobacterium* strain EHA 105 was transformed efficiently with the recombinant plasmids.

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 · l^{-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).

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

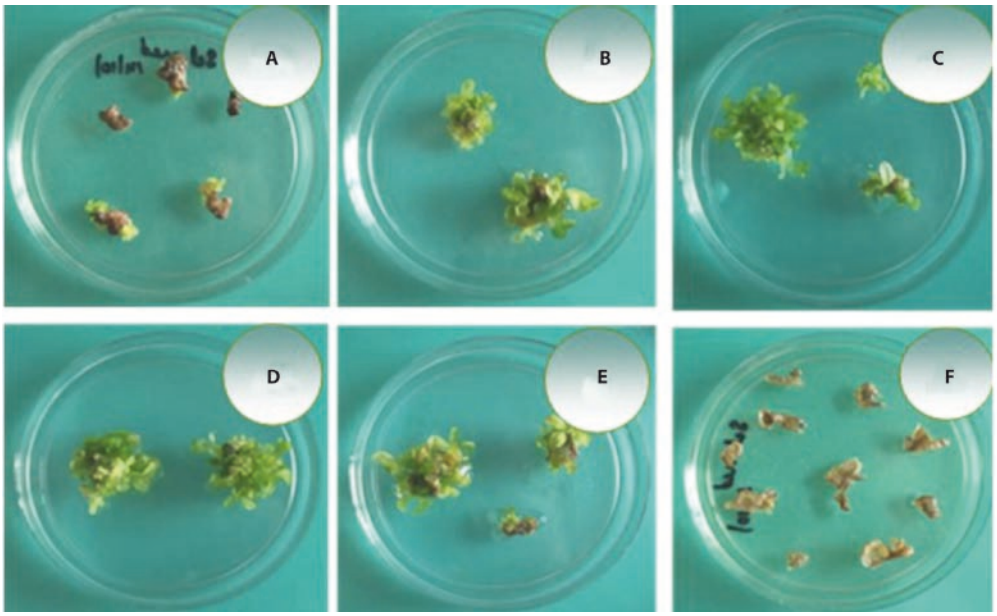

Fig. 5. Regeneration of transformed tobacco plantlets in the selection plate. A – tobacco leaf discs transformed with promoter-less construct; B – tobacco leaf dics transformed with 913 bp promoter constructs; C – tobacco leaf dics transformed with 582 bp promoter constructs; D – tobacco leaf dics transformed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

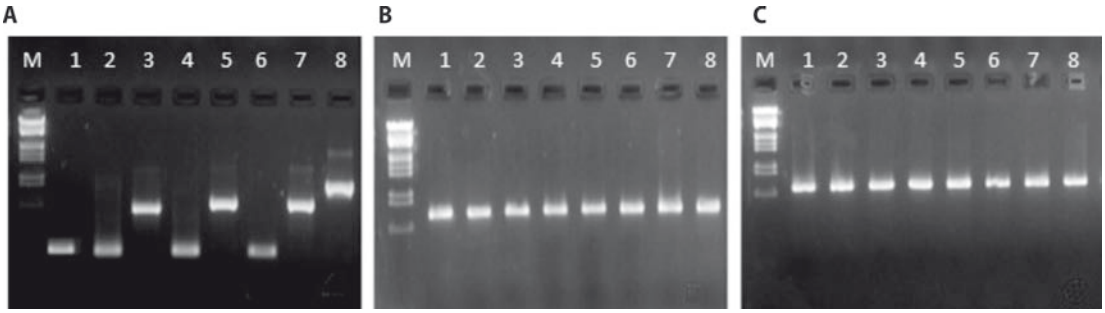

Fig. 6. PCR amplification for confirmation of transformation in transgenic tobacco plants. A – PCR amplification of inserted promoter regions (M – λ DNA *Eco*R1/*Hind*III double digest marker; 1 –198 bp, 2 – 553_{200}, 3 – 553, 4 – 582_{200}, 5 – 582, 6 – 913_{200}, 7 – 913_{550}, 8 – 913 bp); B –amplification of GUS gene amplification from transformed tobacco with the 8 binary vector constructs (M – λ DNA *Eco*R1/*Hind*III double digest marker, 1–8 GUS gene amplified from samples in the order as in A above); C – amplification of *hpt*II gene from transformed tobacco with the 8 constructs (M – λ DNA *Eco*R1/*Hind*III double digest marker), 1–8: *hpt*II gene amplified from samples in the order as in A

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.

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. This

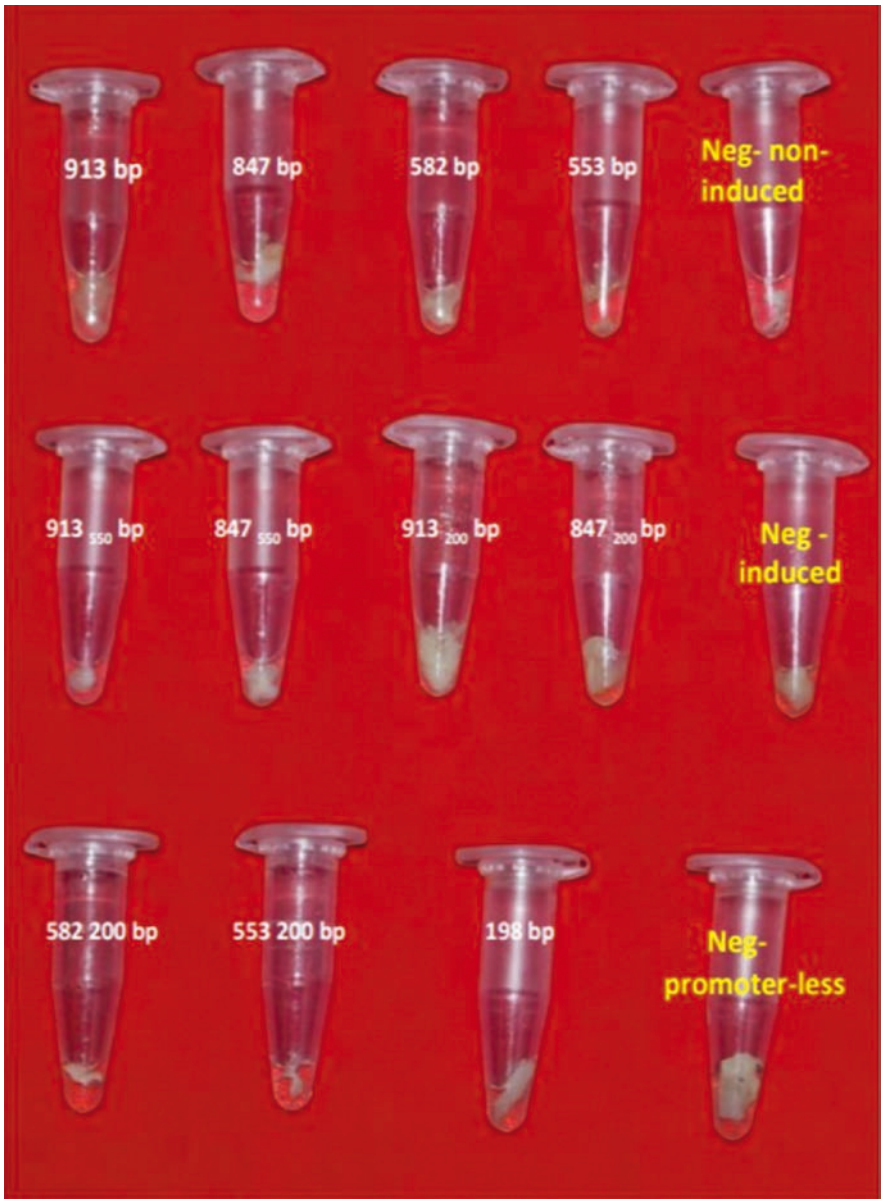

Fig. 7. Transgenic tobacco leaf bits showing no GUS activity after transforming with promoter: GUS fusion binary vectors. Tobacco leaf transformed with the binary vector carrying intact promoters and their 200 and 550 bp deletion constructs along with controls – salicylic acid induced and non-transformed;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

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

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*.

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