

Sequence Analysis and Expression Study of LTP7 Promoter Isolated from Cotton (*Gossypium hirsutum* L.)

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Abstract. Lipid transfer proteins (LTPs) have role in transfer of phospholipids along biological membranes. A cotton LTP7 promoter was isolated using high throughput genomic sequences (HTGS) data base. Analysis of promoter nucleotide sequence revealed a number of crucial regulatory elements including core promoter elements. A 1.8 kb fragment of LTP7 promoter was isolated from genomic DNA of cotton and finally cloned in plant expression vector to characterize its functionality. Transient GUS assay revealed that promoter showed expression in cotton fibres during the time of elongation and different stages of secondary cell wall synthesis. Deletion analysis at 5' end showed that 1 kb promoter showed strong expression during stage of secondary cell wall synthesis. Whereas, 1.5 kb deletion fragment exhibited less strong expression in cotton fibres. Results of this present study, showed that 1 kb deletion fragment and 1.8 kb LTP7 promoter exhibits fibre specific expression and may be used to express fiber genes in cotton.

Keywords: lipid transfer protein, sequence analysis, promoter, transient GUS expression, cotton fibre

Introduction

Fibre development is complex process involving large number of genes that express during specific fibre development phase. Promoters have key role in controlling expression of genes. Upstream regulatory sequences of many cotton fibre genes shows tissue specific or fibre specific expression. Some of LTP genes are expressed distinctly in developing cotton fibres. LTPs are conserved proteins found in epidermal tissues of higher plant. Lipid transfer proteins play role in shuttling of phospholipids and other fatty acid groups between lipid bilayer and responsible for cell wall biogenesis (Allen *et al.*, 2009). These proteins are thought to have role in cuticle deposition and secretory pathway (McGee *et al.*, 1994), plant defense against pathogens attack (Gomes *et al.*, 2003) and plant development (Fleming *et al.*, 1992). Promoters of fibre related genes exhibited strong expression in tobacco, Arabidopsis and cotton fibres (Machando *et al.*, 2009). Many of LTP tobacco plant transformed with homologues of LTP genes promoter showed good expression in leaf, stem trichomes along with vascular tissues and leaf margins (Liu *et al.*, 2000). In order to control fibre specific expression the study of promoter and regulatory region of fibre specific genes is very

helpful. Therefore, need of the hour is to develop cotton varieties with superior quality fibre to avoid foreign exchange as well as to export it to other international market at high prices.

The present study describes isolation and sequence analysis of cotton lipid transfer protein (LTP7) promoter from cotton. Sequence analysis showed important regulatory motifs for promoter activity. Full length 1.8 kb promoter was expressed in cotton fibres. 5' Deletion mutants of 1 kb and 1.5 kb also showed GUS related activity. Although, 1 kb fragment showed strong expression than 1.5 kb fragment. In future they may be able to derive expression of GUS reported gene in cotton fibre. Therefore, LTP7 promoter and its 1 kb promoter may be used to express transgene in cotton fibre to improve cotton fibre quality.

Materials and Methods

An upstream region LTP7 gene from cotton was selected for promoter isolation. Nucleotide sequence of LTP7 was extracted and blast searched in HTGS (high throughput genomic sequences) at NCBI. Nucleotide sequence that were matched with HTGS of cotton was taken and its exact coding frame were identified using ExPasy tool. Two different upstream regions of 2 kb and 1.8 kb were analyzed using protein blast (Blast P) and then both were patent searched in patent blast.

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Finally, about 1.8 kb upstream region of LTP7 gene was chosen for promoter evaluation and characterization. Their transcription initiation site was found by fruitfly database and further bioinformatics analysis was performed using Plant pan and PlantCARE.

LTP7 promoter was isolated from genomic DNA of cotton by PCR amplification using 5' TATGAGCTCGAGTGCAATATTGGCTTACC3' and 5' GACAAGCTTGACTGAAAAATCCACCAAC 3' as forward and reverse primer. PCR product was further cloned in TA cloning vector. Next, they were cloned in expression vector pGR1. To study the expression effect and to study the regulatory motifs 1.5 kb and 1kb promoter were also cloned in pGR1. Their transient expression was analyzed using GUS particle bombardment. Normal promoters and experimental 35S promoter and promoter less constructs were bombarded at same conditions. Cotton tissues bombarded with promoter constructs were dipped in GUS staining solution containing X-Gluc and placed under dark at 37 °C till appearance of blue colour stain. Different cotton tissues were decanted and washed till removal of chlorophyll from green tissues.

Results and Discussion

Based on expression profiling results a highly expressed genes in cotton fibres was selected for promoter isolation, lipid transfer protein (LTP7). Nucleotide sequences of cotton LTP7 promoters were retrieved from high through put genomic sequences (HTGS) in NCBI. BLAST search of LTP gene sequence in HTGS database revealed that sequence matched with HTGS of *Gossypium hirsutum*, clone ZMMBBb 621G9 (Accession no. 202820). For LTP7 promoter 1843 bp (about 1.8 kb) sequence was selected.

Evaluation of promoter sequences using bioinformatics tools. Several bioinformatics tools were used to identify transcription start site and *cis*-acting regulatory sequences of LTP7 promoter. Transcription initiation site was identified by promoter prediction analysis tools available at fruitfly.

TiS site (CTTTTGGCTATAAAAACCCTCCTACCC TCAAGCCCTAACCACGACAATCA)

The promoter sequence of LTP was BLAST searched against patent sequences at NCBI. Results revealed no significant homology with any reported promoter

(Table 1). LTP7 promoter sequence analysis of LTP7 promoter through PlantCARE identified various number of *cis*-acting regulatory motifs (Table 2). In 1.8 kb LTP7 full length promoter, TATA box was identified at position -23. A CAAT box having consensus sequence CAATT was also found at position -131 relative to transcription initiation site. A number of light responsive motifs namely Sp1 motif, G-box (TAGGTG), Box-4 and Box-I were also present in LTP7 promoter.

Similarly to identify various transcription factors plant pan was used, several binding sites for transcription factors were found in LTP7 promoter (Table 3). There were binding sites for MYB proteins that are involved in dehydration response. Similarly two binding sites with consensus sequence CTAACCAC involved in activation of rapid transcription were found in LTP7 promoter.

Isolation of full length cotton LTP7 promoter.

Chromosomal DNA of young cotton leaves was used as template for amplification of full length promoters. Several primer pairs were tested to get amplifications. Gradient PCR for optimized annealing temperature shows that LTP7 promoter was amplified using LTPGh1 (Fig. 1). 8F3 as forward primer and LTPGhR1 as reverse primer at temperature of 48 °C (Fig. 2).

Cloning of LTP7 promoter in TA cloning vector pTZ57R/T.

Amplified product of LTP7 promoters was separately purified and ligated into pTZ57R/T vector then transformed into competent cells (*E. coli*). SacI and ApaI was used for restriction digestion, Two discrete

Table 1. Patent BLAST results for LTP7 promoter

Description	Query coverage (%)	Identity (%)	Accession number
Sequence 38 from patent US 7498428>GZ 935393-1 sequence 38 from patent US812951	32	88	GP23723-1
Sequence 39 from patent US749828>qb GZ 953941 from patent US812951	32	80	GP235724-1
Sequence from patent US 5792933	5	92	AR022671-1
Sequence 17 from patent US 7732678	5	85	GX277307-1

Table 2. *Cis*-acting elements in the LTP7 promoter (plantCARE analysis)

Motif name	Organism	Sequence	Putative function
BOX-I	<i>Pisum sativum</i>	TTTCAAA	Light responsive element
BOX-4	<i>Petroselinum crispum</i>	ATTAAT	Light responsiveness
ABRE	<i>Arabidopsis thaliana</i> <i>Arabidopsis thaliana</i>	TACGTG TACGTG	Abscisic acid responsiveness
ARE	<i>Zea mays</i> <i>Zea mays</i>	TGGTTT TGGTTT	Anaerobic responsiveness
CAAT-Box	<i>Glycine max</i> <i>Glycine max</i> <i>Brassica rapa</i> <i>Arabidopsis thaliana</i> <i>Brassica rapa</i>	CAATT CAATT CAAAT CCAAT CAAAT	Common <i>cis</i> -acting element and enhancer region
G-Box	<i>Antirrhium majus</i>	CACGTA	Light responsiveness
G-box	<i>Daucus carota</i>	TAGGTG	Light responsiveness
MRE	<i>Petroselinum crispum</i>	AACCTAA	MYB binding site involved in light responsiveness
Sp1	<i>Zea mays</i>	CC(G/A)CCC	Light responsiveness
RY element	<i>Halianthus annus</i>	CATGCATG	Seed specific expression
HSE	<i>Brassica oleracea</i>	AAAAATTC	Heat stress responsiveness
MBS	<i>Arabidopsis thaliana</i> <i>Arabidopsis thaliana</i>	CAACTG TAACTG	MYB binding site for drought inducibility
TATA-box	<i>Lycopersicon esculentum</i> <i>Zea mays</i> <i>Lycopersicon esculentum</i>	TTTTA TTTAAAAA TTTTA	Core promoter element around -30 of TSS
TC-rich repeats	<i>Nicotiana tabacum</i>	ATTTTCTCTA	Stress and defense responsiveness
TCA element	<i>Nicotiana tabacum</i>	CCATCTTTT	Salicylic acid responsiveness
5' UTR Py-rich stretch	<i>Lycopersicon esculentum</i>	TTTCTTCTCT	<i>Cis</i> -acting element conferring high transcription

bands of 1.8 kb LTP promoter and 2.8 kb vector backbone were obtained after digestion of recombinant clones (Fig. 3).

Construction of deletion fragments of LTP7 promoter. Full length promoters cloned in TA cloning vector were used as template to generate promoter deletion fragments. Confirmation of clones was carried out through restriction analysis using *SacI* and *HindIII* (Fig. 4).

Important regulatory motifs in LTP7 promoter fragments. Deletion analysis of LTP7 promoter at 5' end resulted in deletion of a number of motifs with diverse important functions (Table 4). Full length LTP7 cotton promoter consisted of 23 binding sites for ARRIAT (aataacacgtaggtgg) whereas its 1.5 and 1 kb deletion fragments consisted of 18 and 11 sites, respectively.

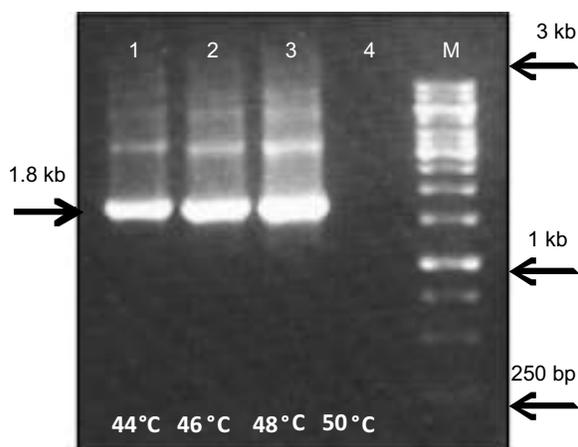


Fig. 1. Gradient PCR for amplification of LTP7 promoter. M: 1 kb DNA ladder; Lane 1-4 represent range of annealing temperatures 44, 46, 48 and 50 °C, respectively.

Cloning of full promoters and their fragments into expression vector (pGR1). Finally the promoters and their deletion fragments were inserted into expression vector pGR1 by substituting the widely used 35S promoter upstream of GUS gene. pGR1 vector was restricted with SacI and HindIII to excise 750 bp 35S promoter fragment. Full length LTP7 promoter and its deletion fragments were amplified through PCR and restricted with SacI and HindIII for ligation into restricted vector pGR1. Resultant clones were confirmed after restriction digestion with SacI and HindIII (Fig. 5). 1 kb LTP7 promoter was named pGRLTP1, 1.5 kb promoter was named pGRLTP1.5, and pGRLTP1.8 having 1.8 kb LTP7 promoter. Physical maps of pGRLTP1, pGRLTP1.5 and pGRLTP1.8 are given in Fig. 6A-C.

Transient GUS expression studies. The main objective of present research was to isolate novel plant promoters to obtain high transgene expression. So, to evaluate and analyze the promoter activity of isolated promoters and their deletion fragments transient GUS assay was performed. Along with full length promoter two deletion fragments of 1.5 and 1 kb were cloned in expression vector pGR1 and their expression was determined through transient GUS assay. Cotton fibre and various tissues were used as explants to determine experimental accuracy along with positive and negative controls. In order to monitor false results, promoter less constructs were bombarded on cotton tissues to get negative results. No GUS expression was detected in any tissues as shown in Fig. 7. pGR1 vector having 35S promoter was taken as positive control. GUS expression was detected

Table 3. Transcription factor binding sites in LTP7 promoter (plantPAN)

Motif	Sequence	Function	Organism
ABRELATERD1	CACGT	Etiolated induced expression	<i>Arabidopsis</i>
ACGTATERD1	ACGT	Dehydration or dark responsiveness	<i>Arabidopsis</i>
AGL3	CTATTTATGG	Regulator of transcription	<i>Arabidopsis/pea</i>
ANAERO1CONSENSUS	AAACAAA	Anaerobic induction	<i>Arabidopsis</i>
ARRIAT	aataatCACGT aggttg	Transcription activation	<i>Arabidopsis</i>
CARGCWBGAT	TTATTATAG	Binding site for MADS protein	<i>Arabidopsis</i>
AtMYB2	CTAACCA	Drought & ABA responsiveness	<i>Arabidopsis</i>
AtMYC2	CACATG	ABA signaling	<i>Arabidopsis</i>
CCAATBOX1	ATTGG	Heat responsiveness	<i>Carrot/Arabidopsis</i>
DPBFCOREDCDC3	ACACATG	Embryo related motif	<i>Soybean</i>
GT1GMSCAM4	TTTTTC	Pathogen/salted induced response	<i>Arabidopsis</i>
MYCATRD22	CATGTG	Dehydration response	<i>Tomato/Lycopersicon</i>
ERELEE4	AATCAAAA	Ethylene responsiveness	<i>Arabidopsis</i>
GAREAT	TTTGTTA	GA induced response	<i>Petunia/Arabidopsis</i>
GATABOX	TATC	High transcription	<i>Arabidopsis</i>
PIF3	aataatCACGTtaggttg	Phytochrome signaling	<i>Arabidopsis</i>
GT1CONSENSUS	GGTAAA	Light responsiveness	<i>Tobacco</i>
INRNTPSADB	TTCAGTTC	Initiation element of transcription	<i>Arabidopsis</i>
MYB1AT	TAACCA	Dehydration response	<i>Arabidopsis</i>
SEF3MOTIFGM	TGGGTT	Light responsiveness	<i>Arabidopsis</i>
MYB2AT	TAACTG	Water stress response	<i>Arabidopsis</i>
REBETALGLHCB21	TATCCG	Phytochrome response	<i>Arabidopsis</i>
RHERPATEXPA7	TCACGT	Root hair specific expression	<i>Potato</i>
SEF1MOTIF	ATATTTAAT	Embryo specific expression	<i>Arabidopsis</i>
SREATMSD	GGATAA	Sugar responsiveness	<i>Potato</i>
SURE1STPAT21	TTTTCTATT	Sucrose responsiveness	<i>Arabidopsis</i>
SORLREP3AT	GTTTGGTG	Light responsiveness	<i>Potato</i>
MYBST1	GGATA	Transcription activation	<i>Arabidopsis</i>
SV40CORENHAN	CTAACCCAC	Enhancer element	<i>Arabidopsis</i>
TBOXATGAPB	CAAAGT	Light responsiveness	<i>Arabidopsis</i>

-1752 TAGATTTACCAGTGAACAAATCTAGAGAAGAAATTAGTCGAACTACATCAATGTCGAGAAGATCGGGTAGAGATAGAAAAG -1673
-1672 ACAATCTGATTTTCAGGGGTATTCGATAGACCTCCAGCAAAGTGTGAAGCAGTGTTTCAGAAATGTTCCCCCGGGCCTAAGTT -1593
-1592 GTCAATATTGTGGAAGATATTCATTTAGAGAATGCAGGACCAAGATGGGGGGCTTTGTTAAAAATGGTGGGGGGTACTA -1513
-1512 TTCATCTTAATTCGAGATTGTCCCGACTTATAAAAAAGATGAATTGGGAACAGAAAAGAAATACAGAGAAAACCATTTC -1433
-1432 CCCAAAAATAGACGTTTCGGGGCAGAACAGTGCTTCTGGGACTACCCCGTTTCGGGGACGAAGGAATCAGTTGGGCGAT -1353
-1352 CAAAGAATAAAGCACCAGCACGTA CCTCCCCCTTTCTAGCAAAGGAAAGAGGTACCGCCTCCCGGAGGAAATTGCTGG -1273
-1272 GACCTTTTCTATCTTTTTTGATGTTAGGTTTATGCAATTGAAAAGACCCCTGGAACCACTCCCTCCCATTTTTTGCCTA -1193
-1192 TGGTTGCATCTAAAAAAAATAACTGTTTGTAGTCCCCTGGAATTTTTAAGAAGTGGGGAAGATAATTAATCCTTCTT -1113
-1112 GTTTTTTCAGTTATTTAGGGTTGTGTTTTGGGTTTTTTCATTTTATAAATGTTGAGAGCTAAATTTATTGGATTATTTAGAA -1033
-1032 TTTGAACTAAAATGAAAAATATTGTAAACATTGAGGAATAATTTGTTGAATTTTAATTTTTATATTTAAGGCCAAATTA -953
-952 TAAATATAGTAAACATTAGATAACTAAATTTATTATTAGAATCCACACTTCTTTAAATTTCAAATTGCTCTTAACATTG -873
-872 CGCGAAGAAAATATTAGTTTTATATAATTACCAAATAAAAAAAAATAAAAAATCAATCAACAAAATAATCTATAAATA -793
-792 GATTTAGAATGTTGCATGATATATATATATATTTAAAAAACCATGCATGTGCTCAATCTTGTGAAGTTTTTTTTTTTC -713
-712 CATTGAAAATACAAAAATAACCATTGTTTATATTTAATATTAGTTCATGGCATAACCGCCCTGAAAAAGGAAAAATT -636
-632 CATGTAATAATATATTTTATAAAAAATTTATATTAAACTAAAATAAAATTTTAGTTGAAATAGTTAAGTTAAAAAGAGTA -553
-552 AAATTATGAGTCATAAGTTTTAAATCTTATTATAGTTTTATTTATAATAAAAAATAAAAAATACATTTATATTTAATATTT -473
-472 GTTATATGAATATTTTAAACAAATTTCAATTAATAGATGCTTGGGTGAATCGTAATACCAATTCAATTAAGACTTAA -393
-392 TATATAAAGTATATATACAAACAATTATAAATCAATTACTTTTAAATAAATGGATCATTCTGACAATAAATGCAGTT -313
-312 ATAAATACATCAAATGTAAATCTCATGTTTATAAGAAAACACCTAGAAAAAGTTAAACCAATATTTGAGTCCTAGCTGT -233
-232 GGAGGCATGATTGAGTGAATCAAATGGACGCTGGTTTTAATTGTATTGAAAGAAAACAATAATCACGTA GGTGGCAGT -153
-152 TGAACATAATTGAATGGTCTCAACTTTAATGTGGTGTGTTAATGTTTGGATCGGATAATCTAACTTACCTAATAGCTA -73
-72 GAAAGTAAAATTCAGACATCACCCGCTACTACTTTTGGCTATAA AAACCCTCCTACCCTCAAGCCCTAACCGACAAT 8
9 CAGCAATAGTACTACTACTCCAAGCAAGCATTTCCTTACAAGTTTGTGTTTCTTGTGATTAATCGATATGGTAGCTCA 88

ATG

Fig. 2. Nucleotide sequence of cotton LTP7 promoter. Translation initiation codon (ATG) of LTP7 gene is highlighted with violet colour and transcription initiation site (+1) has been indicated with letter (A) and dark red colour. TATA box is located at (-27) position indicated by green colour while CAAT box is highlighted by dark grey colour. G-box and ABRE are highlighted with yellow and light grey colours, respectively.

in all tissues of cotton under same experimental conditions (Fig. 8). Cotton fibres, bombardment with LTP7 promoter (1 kb) construct exhibited strong GUS expression (Fig. 9). The transient GUS analysis of 1.5 kb LTP7 promoter showed much weak GUS expression in fibres. No GUS expression was observed in root, stem and leaf (Fig. 10.) The transient GUS analysis of full length 1.8 kb LTP7 promoter showed very strong

GUS expression in fibre tissues. There was no GUS expression in cotton root, stem and leaf (Fig. 11).

Genetic engineering is most powerful tool for introducing genes which are involved in fibre improvement. Cotton fibre is an important food product used universally as raw material for manufacture of fabrics. Molecular breeding approaches are being used to improve quality of fibre for further use in textile industry.

Table 4. Important regulatory motifs in LTP7 promoter, its 1.8 kb, 1.5 kb and 1 kb fragment

Motif name	1.8 kb	1.5 kb	1 kb
ARRIAT	23	18	14
MYBIAT	7	6	5
MYBST1	1	1	1
MYB2AT	3	3	1
ABRELATERD1	2	2	1
CCAAT BOX	5	4	2
GT1GMSAM4	5	5	3
GATA box	9	6	4
WBOXNTERF3	1	1	1
PIF3	1	0	0
DPBFCORED3	1	0	0
MBS	3	2	2
TCA element	2	1	0
AC1 motif	1	1	1
5' UTR pr rich stretch	1	0	0
GAREAT	3	2	1
SREATMSD	1	1	1
TBOXATGAPB	1	1	0
SV40CORENHAN	2	2	1
ERELEE4	1	1	1
ANAERO1CONSENSUS	2	2	1

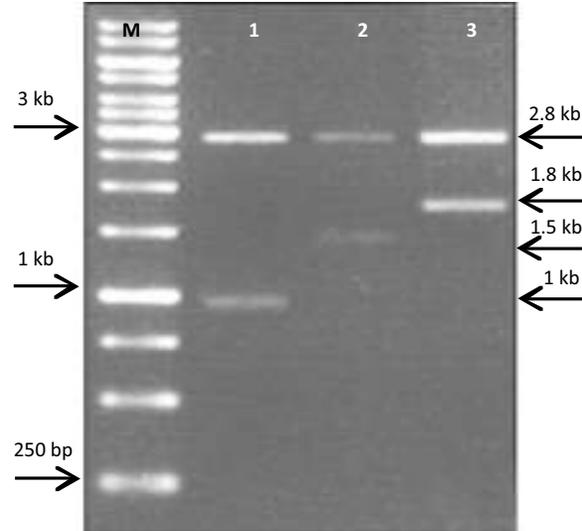


Fig. 4. Cloning of promoter deletion fragments in TA cloning vector. B: Restriction digestion of LTP7 promoter and its fragment with SacI and ApaI. M: 1 kb ladder marker; Lanes 1, 2 and 3 represent 1 kb, 1.5 kb and 1.8 kb LTP7 promoter fragments, respectively along with 2.8 kb vector back bone.

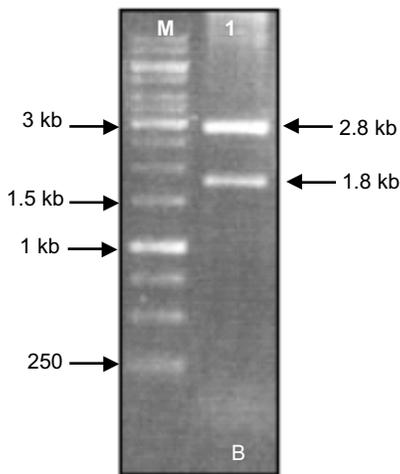


Fig. 3. Restriction digestion of full length LTP7 promoters cloned in TA cloning vector. (A) M: 1 kb DNA ladder; Lane 1 shows restriction digestion of clone using SacI and ApaI releasing 2 kb SPS promoter along with 2.8 kb vector back bone. (B) M: 1 kb DNA ladder; Lane 1: Restriction analysis showing 1.8 kb LTP7 promoter and 2.8 kb vector back bone.

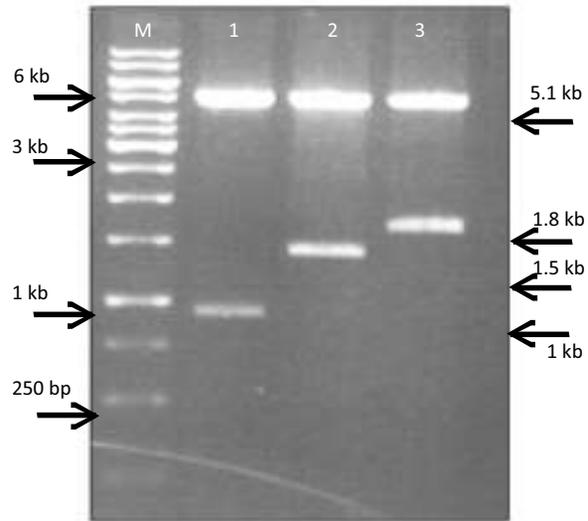


Fig. 5. Cloning of LTP7 promoter and its deletion fragments in Plant expression vector pGR1. Restriction digestion of promoter fragments cloned in pGR1 with SacI/HindIII to confirm clones. M: 1 kb DNA ladder, Lanes 1, 2 and 3 represent 1 kb, 1.5 kb and 1.8 kb LTP7 promoter fragments, respectively along with 5.1 kb vector backbone.

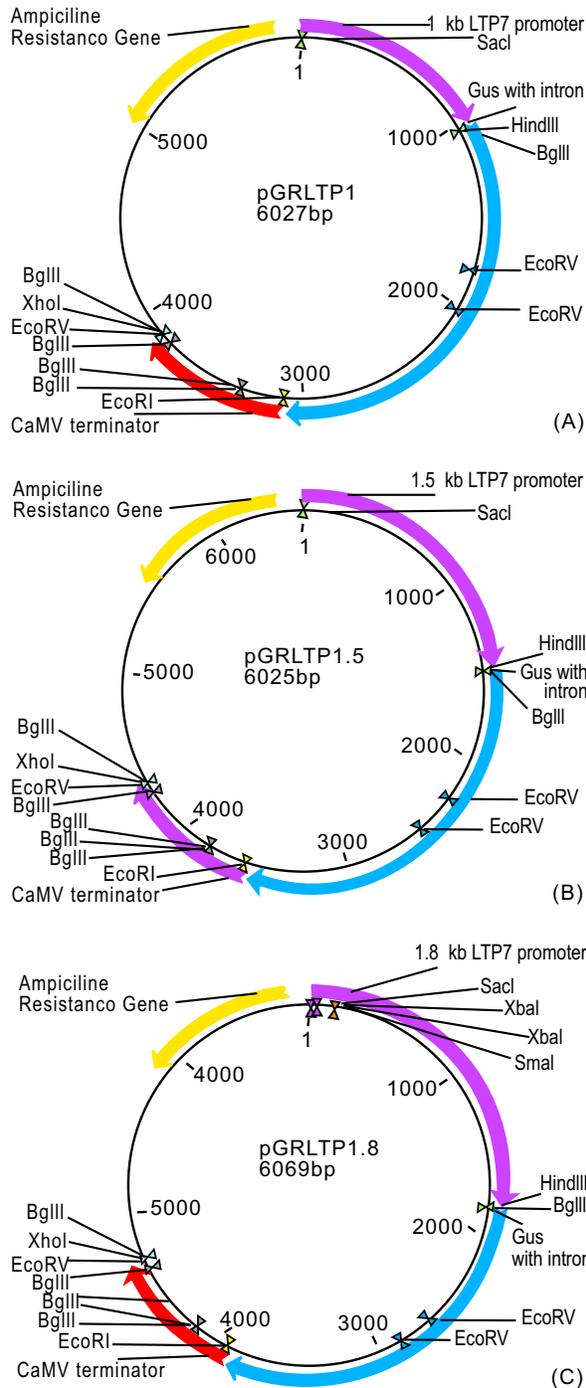


Fig. 6. Vector maps of plasmid constructs containing LTP7 promoter and deletion fragments cloned in pGR1. A: Physical map pGRLTP1 (expression cassette having 1 kb LTP7 promoter). B: Physical map of pGRLTP1.5 (expression cassette having 1.5 kb LTP7 promoter). C: Physical map of pGRLTP1.8 (expression cassette containing 1.8 kb LTP7 promoter).

The focus of present research was to isolate and evaluate novel upstream regulatory sequences of fibre related genes. HTGS sequence of LTP7 was analyzed using bioinformatics based analysis tools approaches. Patent BLAST revealed that nucleotide sequence of LTP7 promoter (Accession no. 202820) had 32% query coverage with previously reported sequence GP23572-1 (Table 1) means these novel sites can be analyzed and evaluated as promoter regions.

Analysis of promoter sequence through plantCARE identified various *cis*-acting motifs in LTP7 promoters (Table 2). In LTP7 promoter TATA box was located at position of -27 bp from transcription initiation site (Fig. 1). Likewise CAAT box motif that is having role in enhancing transcriptional activity (Dai *et al.*, 1999) of promoter was also located at in LTP7 promoters. Endosperm specific elements names as Skn-1 motif

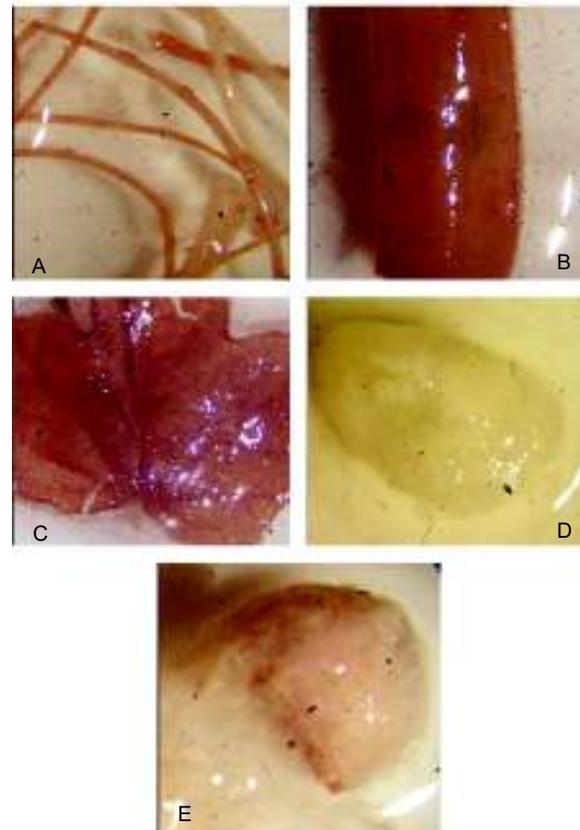


Fig. 7. Transient GUS expression of promoterless construct after bombardment on cotton tissues. (A) root, (B) stem, (C) leaf, (D) 5 DPA fibres and (E) 15 DPA fibres.

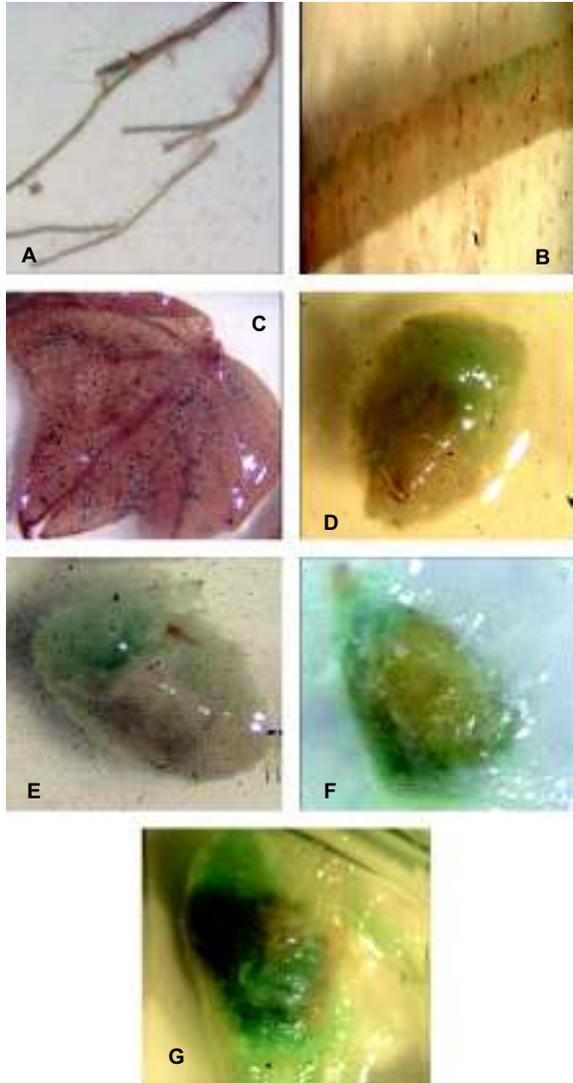


Fig. 8. Transient GUS expression of 35S promoter. Transient GUS expression in cotton tissues bombarded with 35S promoter. (A) root, (B) stem, (C) leaf, (D) 5 DPA fibres, (E) 10 DPA fibres, (F) 15 DPA fibres and (G) 20 DPA fibres.

(GTCAT) and GCN4 motif (TGTGTCA) were also present in LTP7 promoter. Another motif 5' UTR pyrich stretch that shows high gene expression (Daraselia *et al.*, 1996) was also identified in LTP7 promoter. However, an additional seed specific motif named RY element (CATGCATG) was found in LTP7 promoter as described by Yoshino *et al.* (2006). LTP7 promoters also contained two MYB sites that bind specifically with the MYB transcription factors. MYB are the largest

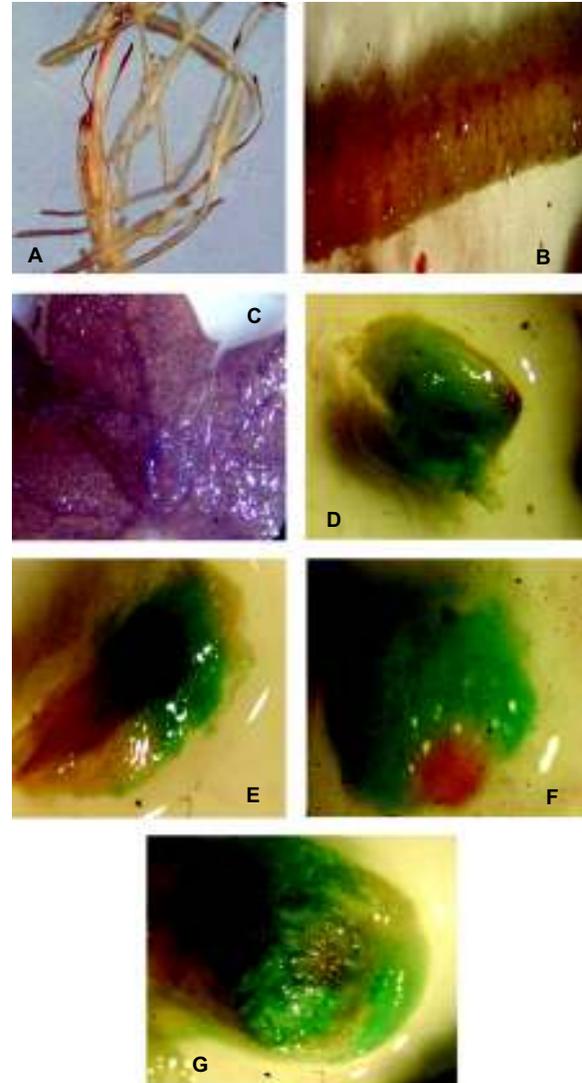


Fig. 9. Transient GUS expression of 1 kb LTP7 promoter. Transient GUS expression of 1 kb LTP7 promoter in different cotton tissues. (A) root, (B) stem, (C) leaf, (D) 5 DPA fibres, (E) 10 DPA fibres, (F) 15 DPA fibres and (G) 20 DPA fibres.

transcription factors family having diverse functions in plants like response to various biotic and abiotic stresses (Yang *et al.*, 2012; ; Kaur *et al.*, 2010; Shangan *et al.*, 2008). In LTP7 promoter several other MYB binding motifs were also identified that shows good expression in dehydration (Abid *et al.*, 2009). In LTP7 promoter TC rich repeat was also found that shows responsive to stress or fungus attack in previously reported promoters (Daiz-De-Leon *et al.*, 1993). A number of other

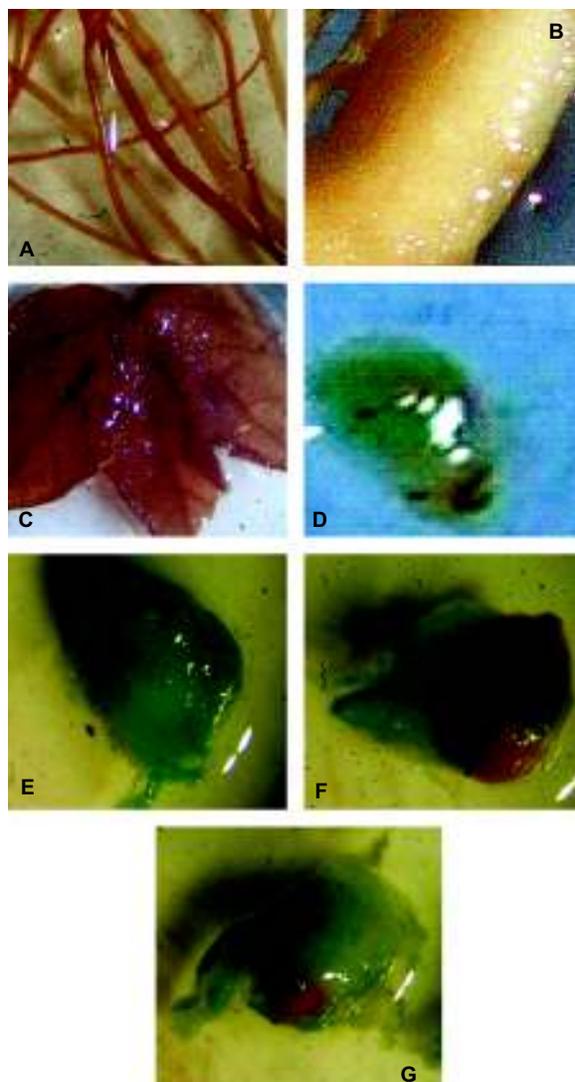


Fig. 10. Transient GUS analysis of 1.5 kb LTP7 promoter. Expression of GUS gene under control of 1.5 kb LTP7 promoter in different cotton tissues. (A) root, (B) stem, (C) leaf, (D) 5 DPA fibres, (E) 10 DPA fibres, (F) 15 DPA fibres and (G) 20 DPA fibres.

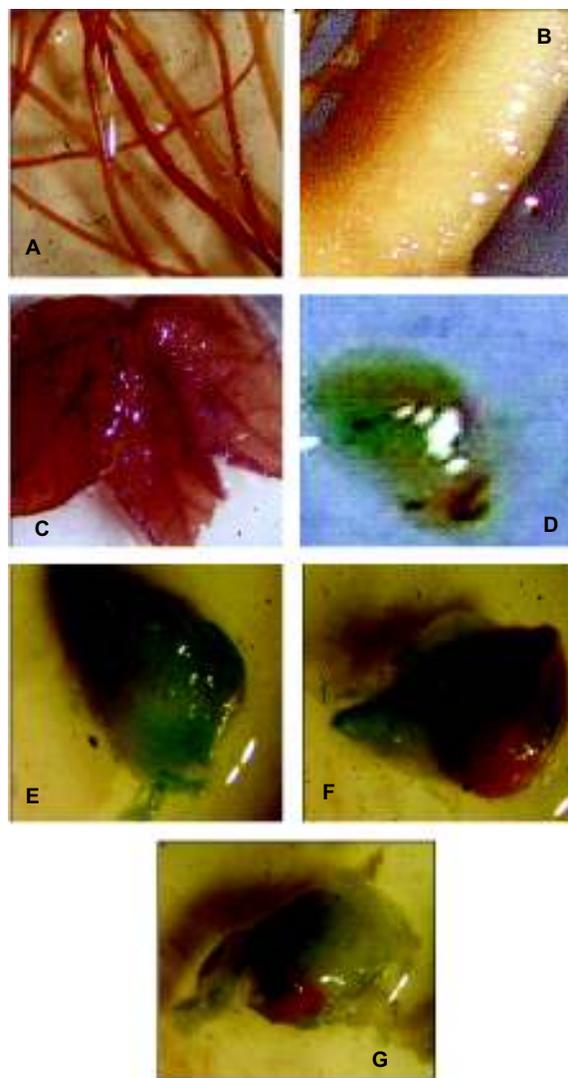


Fig. 11. GUS expression analysis of 1.8 kb LTP7 promoter. Transient GUS expression of 1.8 kb LTP7 promoter in cotton tissues. (A) root, (B) stem, (C) leaf, (D) 5 DPA fibres, (E) 10 DPA fibres, (F) 15 DPA fibres and (G) 20 DPA fibers.

motifs regulated by light including AE-box, box-I, GT-1 motif, G-box and Box-4 were also present in LTP7 promoter (Zhou *et al.*, 2006). G-box is protein binding sequence found in the Rubisco promoter induced by various environmental stimulus (Mary *et al.*, 1992). LTP7 promoter contained additional SORLREP3AT and TBOXATGAPB regions involved in light regulation. A heat shock element with consensus sequence (AAAAAATTC) was also found in LTP7 that was

reported to have role in heat stress responsiveness (Nover *et al.*, 2001). Several number of motifs related to regulation of transcription were present in LTP7 promoter as reported in tomato *ataB* gene promoter (Sakai *et al.*, 2000). SREATMDS and SURE1STPAT21 motifs were also identified in LTP7 that were previously found in cotton glucuronosyl transferase gene (GhGlcAT1) promoter (Wu *et al.*, 2009). Similarly LTP7 motifs including root hair specific motif

RHERPATEXPA7, embryo specific motifs named MYBCORE and DPBFCOREDCDC3 while AP1 was floral organ specific motif. Sequence comparison of LTP7 with previously reported promoters LTP3 and FsLTP4 revealed several common regulatory motif in these promoters like G-BOX, HSE, MRE, BOX-4, BOX-I, and MBS.

For expression studies, promoter fragments were cloned into expression vector pGR1 by replacing 35S promoter upstream of GUS gene followed by CaMV terminator. Biolistic transformation is a useful approach to study tissue specific expression of cotton promoters (Kim *et al.*, 2003). GUS expression pattern after biolistic transformation depicted that LTP7 promoter and its deletion fragments revealed that full length promoter and its 1 kb deletion fragment exhibited strong expression in fibre tissues (Fig. 9-11). However, 1.5 kb deletion fragment showed weak GUS expression (Fig. 10). In non-fibre tissues, no GUS activity was exhibited by 1.8 kb promoter and its both deletion fragments. Deletion of 500 bp resulted in lowering expression in fibre tissues. In full length 1.8 kb single 5' UTR py-rich stretch was present which was deleted in its deletion mutants. Other motif related to phytochrome regulated expression was deleted in 1.5 kb fragment. A number of transcription binding sites related to high transcription including GATA box and MYBST1 were trimmed in 1.5 kb fragment. There may be some negative regulatory region -500 bp away from TiS. This showed that regulatory transcription elements present away from core promoter region also play important role in modulating gene expression as observed 1.5 kb deletion mutant. Surprisingly, strong expression in fibre again appeared in 1 kb fragment. In 1 kb LTP7 promoter although 5' UTR py-rich stretch was deleted but 4 GATA motifs along with SV40CORENHAN and single MYB binding sites were present in this region. Cotton tissues were bombarded with 35S promoter (positive control) and promoter less construct (negative control) to avoid false results. Regeneration in cotton is a time consuming process. Therefore transient GUS assay was used to overcome problems with somatic embryogenesis and regeneration in cotton. This has proved very useful protocol to detect promoter activity in fibres of different developmental ages. Newly isolated LTP7 promoter have wide potential applications for use to express genes in fibre tissues of cotton especially during elongation period that is the most crucial stage of fibre development. Elongation phase determines the length of cotton fibre

that is most desirable for textile industry. Therefore, this promoter may be used to express fibre genes specifically during elongation phase in fibres.

Conflict of Interest. The authors declare no conflict of interest.

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