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Isolation and characterization of gene sequences expressed in cotton fiber¹

Isolamento e caracterização de sequências gênicas expressas em fibras de algodão

Taciana de Carvalho Coutinho², Marcelo de Almeida Guimarães^{3*} and Marcia Soares Vidal⁴

ABSTRACT - Cotton fiber are tubular cells which develop from the differentiation of ovule epidermis. In addition to being one of the most important natural fiber of the textile group, cotton fiber afford an excellent experimental system for studying the cell wall. The aim of this work was to isolate and characterise the genes expressed in cotton fiber (*Gossypium hirsutum* L.) to be used in future work in cotton breeding. Fiber of the cotton cultivar CNPA ITA 90 II were used to extract RNA for the subsequent generation of a cDNA library. Seventeen sequences were obtained, of which 14 were already described in the NCBI database (National Centre for Biotechnology Information), such as those encoding the lipid transfer proteins (LTPs) and arabinogalactans (AGP). However, other cDNAs such as the B05 clone, which displays homology with the glycosyltransferases, have still not been described for this crop. Nevertheless, results showed that several clones obtained in this study are associated with cell wall proteins, wall-modifying enzymes and lipid transfer proteins directly involved in fiber development.

Key words: *Gossypium hirsutum* L.. cDNA Library. Transfer proteins. Arabinogalactan. Glycosyltransferase.

RESUMO - As fibras de algodão são células tubulares que se desenvolvem a partir da diferenciação da epiderme do óvulo. Além de serem uma das mais importantes fibras naturais do grupo têxtil, as fibras do algodão fornecem um excelente sistema experimental para o estudo da parede celular. Este trabalho teve por objetivo o isolamento e a caracterização de genes expressos em fibras de algodão (*Gossypium hirsutum* L.) para serem empregados futuramente em trabalhos de melhoramento do algodão. Fibras da cultivar de algodão CNPA ITA 90 II foram utilizadas para extração de RNA e posterior geração de uma biblioteca de cDNAs. Foram obtidas 17 sequências, dentre estas 14 já se encontram descritas em banco de dado do NCBI (National Center for Biotechnology Information), tais como as codificadoras das proteínas transportadoras de lipídios (LTPs) e das arabinogalactana (AGP). No entanto, outros cDNAs, como o clone B05, que apresenta homologia com as glicosiltransferases, ainda não foi descrito para esta cultura. Porém, os resultados indicaram que vários clones obtidos neste trabalho estão associados a proteínas da parede celular, enzimas modificadoras de parede e proteínas de transferência de lipídeo, envolvidas diretamente no desenvolvimento da fibra.

Palavras-chave: *Gossypium hirsutum* L.. Biblioteca de cDNA. Proteínas de transferência. Arabinogalactana. Glicosiltransferase.

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INTRODUCTION

Most cotton breeding programs in Brazil make use of conventional crossing methods, employing the natural variation of genotypes to transfer desirable phenotypic traits to the progeny (CARVALHO *et al.*, 2003). However, depending on the type of gene action, such procedures can reduce or inhibit the expression of other important characteristics in the parental strains (strength, colouration, length, chemical compatibility, water absorption, thermal properties and resistance), reducing the expectation of the development of promising cultivars (NOVAES *et al.*, 2011).

In recent decades, with the advent of transgenics, several cultivars have been developed containing specific genes that contribute to an increase in the efficiency of plant breeding in various cultivated species, such as soybean which is resistant to the herbicide glyphosate (RR soybean), and maize and cotton that are resistant to insects (BT corn and Bollgard cotton respectively) (CASTRO, 2008).

Currently, the genetically modified crops for marketing in Brazil are: Bollgard I (insect resistant, 2005), Roundup Ready (herbicide tolerant, 2008), Liberty Link (herbicide tolerant, 2008), Bollgard I and Roundup Ready (insect resistant and herbicide tolerant, 2009), Widestrike (insect resistant and herbicide tolerant, 2009), Bollgard II (insect resistant, 2009), GlyTol (herbicide tolerant, 2010), TwinLink (insect resistant and herbicide tolerant, 2011) and MON88913 (herbicide tolerant, 2011) (BRASIL, 2012).

Other characteristics of broad interest for cotton producers are related to fiber properties. In the literature, several studies have identified genes related to the development and expression of the fiber throughout its formation. Such as that described by Suo *et al.* (2003) in which the GhMYB109 cotton gene is expressed during initiation and elongation of the fiber, also encoding the MYB transcription factor, important in the regulation of many developmental aspects of the plant, such as hormonal signalling and cellular metabolism.

Formation of the primary and secondary cell wall is a crucial step in the development of cotton fiber, various genes are involved in the synthesis of the polysaccharides and proteins that are required for the formation of these structures. The isolated genes PCKe6, PCKH6, PFB-B6 and LTP6 are directly involved in cell-wall synthesis and the expansion of fiber cells. Other genes, such as the sucrose synthase gene (Sus) and the actin gene (GhACT1), are activated during the elongation period. The GhACT1 gene

plays an essential role in various cellular processes, including cell division, elongation, differentiation and morphogenesis (WANG; CHEN; XIA, 2005). Another important account of modification of fiber characteristics using transgenics is quoted by Li *et al.* (2004), who introduced the *acsA* and *acsB* genes involved in cellulose synthesis in *Acetobacter xylinum*, and obtained cotton plants whose fiber displayed superior characteristics for length, quality and cellulose content.

Despite the various molecular studies into cotton fiber which have been designed, the identification and characterization of new genes which may be associated with other phenological events is necessary in order to give a better understanding of the processes directly or indirectly related to the fiber.

In view of the above, this work was designed with the aim of identifying and isolating genes involved in the growth and development of cotton fiber (*Gossypium hirsutum* L.), to be employed in future work in cotton breeding.

MATERIAL AND METHODS

Cotton plants (*Gossypium hirsutum* L., cultivar CNPA ITA 90 II) were used for the collection of cotton fiber at 25 days post-anthesis.

The RNA from each sample was extracted using the RNeasy Mini protocol for isolation of the total RNA from plant cells, tissues and filamentous fungi. For the latter protocol the extraction was carried out following the suggestions of the kit manufacturer, using only cotton fiber of 25 days development. The integrity of the RNA was analysed via gel spectrophotometer.

Construction of the fiber cDNA library was begun with the extracted RNA, using the BD Creator Smart cDNA Library Construction kit (BD BIOSCIENCES CLONTECH, 2004) and following the manufacturer's recommendations. The following steps were taken in constructing the libraries: 1) Synthesis of first-strand cDNA, using 1.0 µg RNA; 2) Amplification of the cDNA by LD PCR; 3) Digestion with Proteinase K; 4) Digestion with *Sfi*I; 5) Fractionation of the cDNA; 6) Ligation of the double-stranded cDNA into the pDNR-LIB vector (for this stage, three ligation systems were set up at different cDNA concentrations, 0.5, 1.0 and 1.5 µL); and 7) Transformation of the *Escherichia coli* recombinant plasmid.

The library was evaluated by plating 50 µL of the electroporated cells (produced from the DH10B strain of the bacterium *Escherichia coli*) with the product of

the three ligation systems, onto plates containing LB medium (Luria-Bertani: NaCl 170 mM, bactotryptophane 1%, yeast extract 0.5%, pH 7.5) supplemented with chloramphenicol (25 mg L⁻¹) and 0.8 mg L⁻¹ X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). After selection, the positive colonies were grown on Circle Grow medium with added chloramphenicol (25 mg L⁻¹) for the later miniprep of plasmid DNA, employing a protocol described by Sambrook (1989). The DNAs obtained were digested with BamHI (10 U/ μ l) for clone selection.

The selected clones were sent for sequencing in a Megabase sequencer, using the DYEnamic™ET - Dye Terminator kit. After obtaining the sequences, the alignments were analysed using the ClustalW multiple analysis software (<http://www.genome.jp/tools/clustalw>) to verify homology with the sequences deposited in the Genbank of the NCBI (National Centre for Biotechnology Information), using BLAST (Basic Local Alignment Search Tool).

RESULTS AND DISCUSSION

From the cDNA libraries constructed with RNA from the fiber at 25 days post-anthesis, 96 colonies were sequenced; of these, only 17 gave satisfactory sequencing results (Table 1). The clones which were obtained revealed the presence of expressed genes, as described for cotton fiber and for other species such as *Populus tremula* x *P. tremuloides* (AY935506-1), *Zea mays* (AY112578-1) and *Populus euramericana* (AJ300524-4).

The complexity of the development of cotton fiber suggests that a large number of genes of the species is involved in the stages of initiation, elongation, and fiber maturity. Ji *et al.* (2003) report that about 40 genes related to the development process in cotton fiber have been described, with the genes which have so far been isolated being mainly involved in the stages of expansion and elongation, during which the primary and secondary cell walls are synthesised. ARPAT *et al.* (2004) obtained 46,603 EST sequences, where approximately 14,000 were

Table 1 - BLASTN for the clones obtained after the sequencing reaction. NT - number of nucleotides

Clone	NT	Anotation	Homologous Species	e-value
A09	447	<i>Gossypium hirsutum</i> C312 clone Fb-B6 unidentified fiber mRNA	<i>Mus musculus chromosome 15</i>	0.00
D03	400	<i>Gossypium hirsutum</i> C312 clone Fb-B6 unidentified fiber mRNA	<i>Homo sapiens</i>	2e-137
B01	459	<i>Gossypium barbadense</i> fiber protein Fb8-like mRNA	<i>Debaryomyces hansenii</i>	3e-143
E02	493	<i>Gossypium barbadense</i> fiber protein Fb8-like mRNA	Human DNA sequence	2e-150
H05	424	<i>Gossypium barbadense</i> fiber protein Fb8-like mRNA	<i>Oreochromis mossambicus</i>	2e-128
H07	427	<i>Gossypium barbadense</i> fiber protein Fb8-like mRNA	<i>Mus musculus</i>	2e-82
B07	255	<i>Gossypium barbadense</i> FbLate-2 gene	<i>Debaryomyces hansenii</i>	5e-82
B08	413	<i>Gossypium barbadense</i> FbLate-2 gene	<i>Homo sapiens</i>	3e-103
C01	252	<i>Gossypium barbadense</i> FbLate-2 gene	<i>Homo sapiens chromosome 11</i>	3e-43
C11	140	<i>Gossypium barbadense</i> FbLate-2 gene	<i>Vibrio cholerae</i>	1e-10
C12	356	<i>Gossypium barbadense</i> FbLate-2 gene	<i>Homo sapiens</i>	1e-80
E07	368	<i>Gossypium hirsutum</i> cultivar Siokra 1-2 lipid transfer protein precursor (LTP) mRNA	<i>Gossypium barbadense</i>	4e-173
G06	384	<i>Gossypium hirsutum</i> arabinogalactan protein mRNA	<i>Oreochromis mossambicus</i>	2e-73
H08	319	<i>Gossypium hirsutum</i> arabinogalactan protein mRNA	<i>S.cerevisiae</i>	9e-78
B05	435	<i>Populus tremula</i> x <i>Populus tremuloides</i> secondary cell wall-related glycosyltransferase family 47 mRNA	<i>Arabidopsis thaliana</i>	2e-27
G05	430	<i>Zea mays</i> CL885_1 mRNA sequence	<i>Oryza sativa</i>	1e-22
H04	308	<i>Populus euramericana</i> mRNA for putative dehydrin (dhn1 gene)	<i>Phaseolus vulgaris</i>	1e-15

genes associated with the development of cotton fiber, meaning that 35-40% of the genome of the species appears to be directly or indirectly related to fiber development.

Among the genes already characterised in cotton and identified in this study is the gene for the lipid transfer protein (LTP), which has 368 nt and an e-value of $4e-173$. The LTPs are a group of small proteins containing from seven to eight conserved cysteine residues, known as lipid transfer proteins. In general, they are extracellular proteins with signal peptides for localisation on the cell wall and other cellular layers. Similar to other plants, Ltps genes (631 nt) have an open reading frame interrupted by a single intron of 136 bp, and are located in the C-terminal region of the protein (ALTSCHUL *et al.*, 1997). The cDNA sequence (E07) isolated for LTPs displayed significant similarity (97%) to the gene encoding the isolated LTP protein in the Siokra cultivar of cotton.

Other genes described for cotton were: Fb-B6, Fb8, FbLate-2 and arabinogalactan. For the Fb-B6 gene, two sequences were isolated, named A09 (447 nt) and D03 (400 nt). The sequences displayed 97% identity with the A09 clone and 99% with the D03 clone. In studies carried out by John (1995), the presence of B6 RNA was detected during the stages of fiber development in the synthesis phase of the primary and secondary cell wall. However, the specific function of the gene does not seem to be known.

The cDNA sequences (B01, E02, H05 and H07) shared identity with the Fb8 gene. This gene has a nucleotide sequence of 913 nt, whereas the sequences isolated in the present work have fragments with a varying number of nucleotides, with the E02 sequence showing the greatest number of nucleotides (493 nt), and the smallest sequence being H05 with 424 nt. The E02 and H05 clone sequences displayed an identity of 91%, while B01 displayed 90%, and H07, 88%. According to Liu *et al.* (2006), the Fb8 gene plays an important role in cellulose synthesis, the main polysaccharide in cell-wall formation.

For the FbLate-2 gene, which shows a nucleotide sequence of 1699 nt, five cDNA sequences were isolated (B07, B08, C01, C11 and C12). These sequences displayed an identity with the FbLate-2 gene of 94% in relation to B07, 92% with B08 and C11, 89% with C12, and 87% with C01. The largest isolated sequence was for the B08 clone, with 413 nt. The FbLate-2 gene is activated during fiber development, a stage where a large amount of cellulose is synthesised and deposited in the secondary cell wall. However, the exact role of the FbL2A gene in the development of the secondary wall of the developing fiber is still being clarified (RINEHART; PETERSEN; JOHN, 1996).

According to Rinehart, John and Petersen (1996), it is unlikely that the FbL2A gene plays any role in the formation of the primary cell wall, since the gene is more active at the end of the development phase of the cotton fiber. It is important to highlight the presence of the FbL2A protein at the start of fiber dehydration, possibly developing a structural role in the secondary cell wall of the fiber.

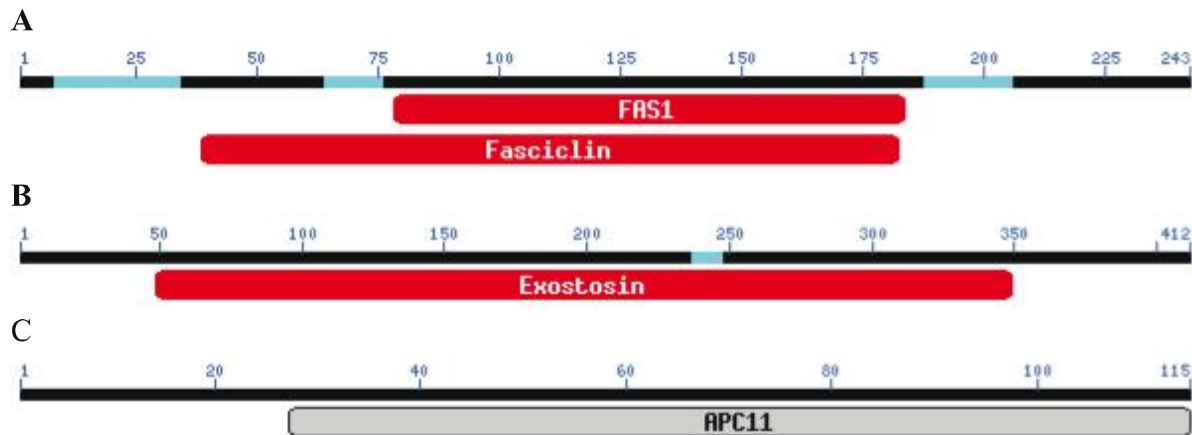
Two other sequences with different sizes were isolated for cotton fiber, the H08 and the G06 clones; the first sharing an identity of 98%, and the second of 99% with the protein arabinogalactan, described for *Gossypium hirsutum* L., presenting e-values of $9e-78$ and $2e-73$ respectively. From the alignment, the presence of two domains for the H08 clone protein was found, the FAS1 domain and the fasciclin domain (Figure 1A). The arabinogalactan proteins (AGPs) comprise a diverse group of plant proteoglycans. In general, they are polysaccharides synthesised during the formation of the plant cell wall, generally classified into types I and II. AGPs are involved in various aspects of the growth and development of plant cells. Recent studies using mutants demonstrated the role of AGPs in cell expansion, coordination of vascular development and development of the cotton fiber (POON; HEATH; CLARKE, 2012).

From the *in silico* analysis of the isolated sequences it was possible to detect that of the 17 sequences isolated, 14 shared identity with genes characterised in cotton. Of the five genes that share identity with the isolated sequences, four (Fb-B6, Fb8, FbLate-2 and arabinogalactan) are involved in cell wall synthesis, and one is related to lipid transfer (LTPs). On this point, it should be noted that during the 25-day post-anthesis period, when the cDNA library was constructed, the cotton fiber is generally in a period of elongation, in which the cells display a vigorous expansion, with peaks in growth of greater than 2 mm day^{-1} ; the cell wall is also being synthesised (JI *et al.* 2003). This is, therefore, a decisive step in the development of the cotton fiber, in which several genes are involved in the synthesis of the polysaccharides and proteins that are required for the formation of these structures.

As previously mentioned, the existence of clones that share identity with proteins already described for other species, such as the B05, H04 and G05 clones, has also been verified.

The B05 clone shares 83% identity with a glycosyltransferase belonging to family 47, described for *Populus tremula* x *P. tremuloides* (ASPEBORG *et al.*, 2005), with an e-value of $2e-27$. The glycosyltransferases, together with the glycosidases, comprise a group of enzymes of great importance in

Figure 1 - A. FAS1 and fasciclin domains of the arabinogalactan protein described for *G. hirsutum*. B. Ring-box protein-like APC11 domain described for *A. thaliana*. C. Exostosine domain of the glycosyltransferase family 47 described for *Populus tremula* x *Populus tremuloides*



the biosynthesis and remodelling of carbohydrate polymers of the cell wall during the different stages of plant development (ASPEBORG *et al.*, 2005).

During analysis of the alignment between protein translated from the nucleotide sequence of this clone (nt 457) and protein described for *Populus tremula* x *P. tremuloides*, it was found that the protein sequence of 67 amino acids, deduced from the B05 clone used in the alignment, corresponds to the C-terminal region of the

glycosyltransferase protein, which has 412 amino acids (Figure 2). In animals, the glycosyltransferases belonging to family 47 (GT47) are part of the amino terminal domain of the heparan synthases, being responsible for the addition of β -1.4-GlcA residues, while the second domain, belonging to family GT64, is responsible for the addition of α -1.4-GlcA residues. The equivalent in plants has a single domain, that of the exostosins (KAMRA; GOKHALE; MOHANTY, 2005) (Figure 1b).

Figure 2 - Alignment between the GT47 protein in *Populus tremula* x *P. tremuloides* and the deduced protein in the B05 clone sequence

>secondary cell wall- related glycosyltransferase family

MR T C L W V F A L V L V F G F V D G K K I E R L R T E R I S G S A G D V L D D D P V G R L K V Y V
Y E L P S K Y N K K L L Q K D P R C L T H M F A A E I F M H R F L L S S P V R T L N P D E A D W F Y
S P I Y P T C D L T P M G L P L P F K S P R M M R S A I Q L I S S N W P Y W N R T E G A D H F F V V
P H D F G A C F H C Q E E K A V E R G I L P L L Q R S T L V Q T F G R R N H V C L N E G S I T I P P
F A P P Q K M Q A H Q I P P D I P R S I F V Y F R G L F Y D V N N D P E G G Y Y A R G A R A A V W E
N F K N N P L F D I S T D H P T T Y Y E D M Q R A I F C L C P L G W A P W S P R L V E A V V F G C I
P V I I A D D I V L P F A D A I P W E E I G V F V A E E D V P N L D T I L T S I P P E V I **L R K Q R**
L L A N P S M K R A M L F P Q P A Q P G D A F H Q I L N G L A R K L P H D R S V Y L K S G Q N I L N
W T A G P V G D L K P W

>Clone B05

LLANPSMKRAMLFPQPALPGDAFHQILNGLARKLPHQKSIYLPGEKILN
WTAGPVGDLKPW

The H04 clone shares 92% identity with a putative dehydrin protein (gene *dhn1*) described for *Populus tremula* x *Populus tremuloides*, with an e-value of $1e-15$. No domains were found with the *in silico* analysis of the isolated sequence. However, Rodriguez, Svensson and Malatrasi (2005) report the presence of three domains for dehydrin, known as segments K, Y and S. The dehydrins have been identified as one of the five major classes of LEA proteins (Late Embryogenesis Abundant). Known as family D-11, dehydrins are encoded by a multigene family whose induction is caused by environmental stress, such as dehydration, low temperature, drought and salinity (YAO; LOCKHART; KALANACK, 2005). The *dhn1* gene shows a nucleotide sequence of 1024 nt (RODRIGUEZ; SVENSSON; MALATRASI, 2005), whereas the H04 clone shows only 308 nt.

The G05 clone displayed an 86% shared identity with the ring-box protein-like Rbx1 protein in maize, described for *Arabidopsis thaliana*, with an e-value of $1e-22$. The APC11 domain for the Rbx1 protein was detected from the alignment that was carried out (Figure 1.C). The Rbx1 protein participates in the process of ubiquitination (or the conjugation of ubiquitin). According to Weissman (2001), this process consists of a reversible post-translational modification which results in the formation of an isopeptide bond between the ubiquitin and the protein-substrate, and includes the action of at least three classes of enzyme: E1 (ubiquitin-activating enzymes); E2 (ubiquitin-conjugating enzymes) and E3 (ubiquitin-protein ligases). Ubiquitin is a globular protein highly conserved in eukaryotes, composed of 76 amino acids. The conjugation of ubiquitin to other proteins is essential for the degradation of proteins that are primarily regulated in response to changes in the cellular environment (WEISSMAN, 2001).

Several genes, isolated from cotton fiber, have been characterised, and act on different molecular mechanisms for regulating development of the fiber, such as microtubule formation, signalling proteins, and formation of the primary and secondary cell wall; all preferentially expressed during the development stages, which range from 10 to 25 days post-anthesis. Some of these genes, GHTUB1, GHGLP1, GHMYB, PCKE6 ACP, PCKH6, PFB-B6 and LTP6, display homology with genes expressed in trichomes of *Arabidopsis thaliana*, a species which is considered a model system for the study of gene regulation in plants. Trichomes are unicellular or multicellular hairs, distinct from the leaf and stem epidermis, with perpendicular expansion and a length of approximately 500 μm . The morphological structure is varied, with unbranched tips, or tips with from two to five branches. Their function is mainly to protect the plant

from insect attack, UV radiation damage and for tolerance to drought (KÄRKKÄINEN; AGREN, 2002). For many authors, cotton fiber are considered to be trichomes, since they are single cells derived from the epidermis.

CONCLUSIONS

1. The sequences obtained in the present study are involved primarily in the process of cell wall formation;
2. Despite some obtained sequences already having been described for cotton fiber, the B05 clone, which displays homology with the glycosyltransferases, has still not been described for this crop.

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