Identification of genes associated with Egyptian cotton fiber development using combination of SSH, Microarrays and Real Time RT-PCR

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ABSTRACT

One of the major limitations in the application of genetic engineering or conventional breeding methods in improving cotton fiber is the paucity of information about fiber related genes. Availability of Gossypium barbedense Giza 88 extra-long staple cotton fiber traits provides a unique opportunity to study fiber-associated genes because of its high-quality fiber compared to Giza 90 long staple fiber. To understand the molecular basis of cotton fiber development, we used the combination of suppression subtractive hybridization (SSH), microarrays and real-time reverse transcription-polymerase chain reaction (RT-PCR) technologies to identify the potential genes related to cotton fiber development. Utilizing mRNAs from 15 days post anthesis (dpa) fibers, we constructed a SSH cDNA library from Giza 88 extra long staple fiber as the tester and Giza 90 long staple fiber as the driver. The SSH cDNA library was then screened using microarrays. Microarrays analysis showed that 20 genes were differentially expressed in Giza 88 15-dpa fiber compared to Giza 90 as confirmed by real time RT-PCR. These genes include two beta-tubulins, an actin, a putative kinesin light chain, a cellulose synthase, glycosyl hydrolase family protein, pyruvate decarboxylase, glycoside hydrolase family, GDP-mannose pyrophosphorylase, dynamin-like protein, annexin and a number of genes involved in signal transduction, and protein, nucleic acid metabolism and lipid metabolisms.

Keywords: *Cotton fiber genes; SSH; microarrays; real time RT-PCR.*

INTRODUCTION

Cotton (*Gossypium*) is the leading natural fiber crop and the second most important oilseed in the world (Basra and Malik 1984). Egyptian cotton varieties (*Gossypium barbadense*) cultivated across the Nile Valley, upper Egypt, is characterized with the long staple development in all its varieties such as Giza 90 and the Delta region is characterized with the cultivation of the extra long-staple cotton varieties such as Giza 88.

Traditional cotton breeding methods have faced many challenges and reached recently a plateau stage in fiber improvement. Genetic engineering is a highly desirable approach to avoid the difficulties in classical breeding methods in cotton fiber improvement. Identification and characterization of cotton fiber-associated genes are critical for the fiber improvement strategy through molecular approaches. Cotton fibers are differentiated seed epidermal cells. Cotton fiber growth traditionally occurs in four phases: initiation,

elongation, secondary cell wall thickening, and maturation, and each of the developmental phases overlaps the succeeding phase (Basra and Malik, 1984; Wilkins and Jernstedt, 1999). Fiber initiation begins just one or two days before anthesis and involves the initial protrusion and isodiametric expansion of the epidermal cell above the surface of the ovule. The elongation of cotton fiber starts at anthesis and lasts for about 25 dpa in most cases (Wilkins and Jernstedt, 1999). Fiber quality is based on particular physical and chemical characteristics that are genetically controlled. Many genes from developing cotton fiber have been isolated and characterized, although the function is known only for some of the genes (Kim and Triplett, 2001; Wilkins and Jernstedt, 1999).

The most widely used methods for gene identification and isolation often relay on examining differential gene expression in varied experiments under varied growth conditions. Patterns of gene expression can be compared using differential hybridization screening (Tedder *et.al.*, 1988), represent-tational difference analysis (RDA) (Lisitsyn *et al.*, 1993), serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995), differential display (DD) (Liang and Pardee, 1992) and subtractive library construction (Hedrick *et al.*, 1984). Such techniques are tedious, labor-intensive and time-consuming. Some require large amount of mRNA (cDNA), have high levels of false positives, or are biased for high copy number mRNA. The application of SSH cDNA microarrays and real time RT-PCR technology offers the possibility of providing a rapid, high throughput method to identify differentially expressed genes. Isolation and characterization of the genes differentially expressed in Giza 88 extra-long staple cotton fiber would provide useful information to understand gene function in cotton fiber development. Here, we utilized SSH, cDNA microarrays and real time RT-PCR as a means of identifying differentially expressed genes in Giza 88.

MATERIALS AND METHODS

Plant materials

Plants of *G. barbadense* Giza 90 and Giza 88 were grown in the field in two seasons 2006 and 2007 in Giza station, the Agricultural Research Center. Flowers were tagged, and dated the day they opened as zero day post anthesis (dpa) and collected on 15 dpa. Ovules were removed and frozen immediately in liquid nitrogen. The fiber layer was then scraped from the developing ovule with a scalpel and a forceps under liquid nitrogen and stored at -70°C. Nucleic acid isolations, SSH-cDNA analysis did take place at the Agricultural Genetic Engineering Research Institute (AGERI), real time RT-PCR, and microarrays analysis took place in the Department of Natural Resources and Environmental Sciences, Alabama A&M University, USA.

RNA isolation

Total RNA of Giza 88 and Giza 90 was extracted from 15-dpa fibers according to the previously described method (Wan and Wilkins 1994). Poly (A)+ mRNA was isolated from the total RNA with an mRNA Purification Kit (Qiagen) according to the Vendor's instructions.

Construction of SSH cDNA library

The PCR-Select cDNA subtraction Kit (Stratagene) was used to generate the SSH cDNA sequences according to the vendor instructions. Different preparations of RNA samples from Giza 88 and Giza90 fibers were pooled, respectively, to obtain a broad range of differentially expressed genes. The cDNA was reversely transcribed from 2μg of fiber mRNA of Giza88 and Giza90, respectively. SSH library was constructed using Giza88 fiber

cDNA as tester and Giza 90 fiber cDNA as driver. Tester and driver cDNA were digested with Rsa I, extracted with phenol/chloroform, precipitated with ethanol, and re-suspended in water. The digested tester cDNA was ligated with different adaptors provided in the cDNA subtraction kit. Two rounds of hybridization and PCR amplification were conducted to enrich the differentially expressed cDNA sequences. The final PCR products were purified, inserted directly into the T/A cloning vector and transformed into *E. coli* TOP10 cells using the TOPO TA Cloning Kit (Invitrogen) producing the SSH cDNA library.

PCR amplification of cDNA inserts

Three hundred cDNA clones were randomly selected from the SSH library.The clones were grown overnight at 37°C and then, amplified with M13 forward and reverse primers complementary to sequences flanking the cloning site. The amplification of cDNA inserts was carried out in GeneAmp PCR System 9700 (Applied Biosystems). The 30 µl PCR reaction mixtures contained 18.8 µl of water, 0.3 µl of each M13 forward and reverse primers (10 µM each), 6.7 µl of 5 X Taq buffer, 3.0 µl of dNTP mix (2.5 each), 0.2 µl (1 unit) of Taq DNA polymerase (Promega) and 1 µl of bacterial culture. The clones were denatured at 94 °C for 5 min, followed by 30 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 2 min, with final extension at 72 °C for 5 min. PCR fragments were analyzed on 1% agarose gel electrophoresis, purified using QIAquick PCR Purification Kit (Qiagen) and then, quantified using a spectrophotometer.

cDNA microarrays slide preparation

cDNA microarray slide preparation and microarray analysis for the selected SSH cDNA clones were carried out in the Genomics Core Facility, University of Alabama at Birmingham, AL, USA. The PCR products from the SSH cDNA clones were spotted onto Ultra GAPs slides (Corning Biosciences, Corning, NY, USA) using an OmniGrid 100 contact printer. On the average, 200-300 pg of the amplified SSH fragments were spotted. Each cDNA clone was printed in triplicate in 3X SSC as the printing solution.

Microarrays hybridization and analysis

Total RNA isolated from Giza90 and Giza88 cotton fibers were labeled with Cy Dyes using the Cy3/Cy5 labeling kit as the manufacture instructions (Mirus Inc., Madison, WI, USA). Giza 90 RNA was labeled with Cy3 and Giza 88 RNA was labeled with Cy5. One dye flip experiment was conducted to correct for any dye bias. The independent labeling reactions were combined and concentrated using the Microcon YM30 columns (Millipore, Bedford, MA, USA). Prior to hybridization the slides were rehydrated in 3X SSC, 0.1% SDS at 65°C. for 5 min followed by a rinse in water, then in 95% Ethanol and spun dried at 50 g for 2 min. The slides were crosslinked by UV at 200mJoules in a Stratalinker (Stratagene, LaJolla, CA, USA). Prehybridization of the slides was done in 25% Formamide, 5 X SSC, 0.1% SDS and 1% BSA at 48°C. for 60 min. The slides were dipped in water followed by isopropanol and spun dry at 50g for 2min. Hybridization buffer (2 X, 20 µl) was added (10X SSC, 50% Formamide, 0.2%SDS), and the probes were heated to 70°C. for 5 min to remove any secondary structure and then equilibrated to 48°C. for 1 min. The labeled probes were applied to the slides and covered with a lifter slip, placed in a hybridization chamber at 48°C. for 48 hr. Post-hybridization washes consisted of placing the slides in a Copeland jar containing 1 X SSC and 0.2% SDS at 48°C. to gently remove the cover slip, followed by gentle agitation for 4 min in the same solution. The slides were 184 transferred to another Copeland jar containing 0.1 X SSC,

0.2% SDS at 25°C and agitated again for 4 min, then transferred to 0.1X SSC and agitated for additional 4 min. This wash was repeated once. Finally, the slides were dipped in 0.01X SSC and spun dry at 50 g for 2 min. The dried slides were scanned using an Axon 4000B scanner and the resulting data were analyzed with GeneTraffic (Stratagene) software. Lowess (Global) Normalization Method was utilized to normalize the ratio values. Genes with the mean of normalized $\log_2$ intensity ratio of >1 were identified as differentially expressed genes.

DNA sequence analysis

The identified genes were sequenced using automated sequencers (ABI Prism 3100, Applied Biosystems). These genes were then annotated by sequence similarity searches compared to the GenBank non-redundant (nr) protein database using the BLASTX program. cDNAs with BLASTX e-values < 1e-10 were designated as having significantly similar sequence, and the higher score affirmed the corresponding gene. Functional categories of the identified genes were assigned based on the Gene Ontology annotations (http://www.geneontology.org/).

Real time RT-PCR

Real time RT-PCR was employed to validate the relative change in the expression of genes identified by microarray analysis. The RNA samples from Giza 88 and Giza 90 initially isolated for the microarray analysis were used for real time RT-PCR. Total RNA (2 µl) from each sample was treated with DNase 207 I (Promega) and used for cDNA synthesis. The first-strand cDNA synthesis was performed with Oligo (dT) primer using RETROscript Reverse Transcription for RT-PCR Kit (Ambion, Inc.). The cDNA was diluted with 1:5 for real time PCR reactions which were carried out in 384-well in the LightCycler 480 (Roche Diagnostics Corporation, Basel, Switzerland) using SYBR Green I Master dye (Roche Diagnostics). Gene-specific primers were designed using Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Each real time PCR reaction (20 µl) contained 8.2 µl of water, 0.4 µl of forward and reverse primers, respectively, 10 µl of 2 X SYBR Green I Master and 1 µl of the diluted cDNA. The amplification program consisted of 1 cycle of 95°C for 5 min for Pre-Incubation, followed by 45 cycles of 95°C for 10 sec, 58°C for 10 sec and 72°C for 15 sec. After amplification, a melting curve analysis was run using the program for one cycle at 95°C for 5 sec, 65°C for 1 min and 97°C with 0-second hold in the step acquisition mode, followed by cooling at 40°C for 10 sec. A negative control without cDNA template was run with each analysis to evaluate the overall specificity. To normalize the total amount of cDNA in each reaction, cotton alpha-tubulin gene was co-amplified as the internal control. Each sample was replicated three times and the resultant data were analyzed using LightCycler 480 Relative Quantification Software.

RESULTS AND DISCUSSION

SSH library, microarrays and real time RT-PCR

SSH cDNA fragments were obtained after two rounds of subtraction. The cDNA fragments ranged from about 300 bp to 1.3 kb with most fragment distributed between 400 bp to 800 bp (Fig. 1). The resulting SSH cDNA library then was screened using microarrays. Three hundred SSH clones were randomly picked from the SSH library for microarray analysis. Twenty genes differentially expressed in Giza 88, 15-dpa fibers were identified after microarray analysis (Table 1). All of the differentially expressed genes were functionally annotated by blasting against the GenBank nr protein database, and classified

into 9 functional categories based on their putative functions. The functional classification showed that 8.3% of the identified genes were related to cytoskeleton, 5.5% related to cellular structure and organization, 11.1% related to energy/carbohydrate metabolism, 33.4% involved in protein metabolism, nucleic acid and lipid metabolism, transport and signal transduction, and the remaining genes were of unknown function (27.8%) or had no similarity with known accessions GenBank nr protein database (13.9%) (Table 1). The microarray analysis data for the differentially expressed genes were verified using real time RT-PCR. In some cases, a much higher fold change was obtained from real time RT-PCR than that from microarrays (Table 1) because microarray has low concentration of cDNA resulting in a relatively low dynamic range (Ozturk *et al.*, 2002). The results of real time RT-PCR agreed with the microarray data (Table 1). SSH is a powerful technique to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. The SSH technique requires only one subtractive hybridization round to remove the cDNAs common to both compared populations and thus, can achieve greater than 1000-fold enrichment for differentially expressed cDNAs (Diatchenko *et al.*, 1996, and Wu Z.,_*et al.*, 2005). Ji *et al.*, (2003) applied SSH, coupled with cDNA array, to isolate 172 genes significantly up-regulated in elongating cotton fiber. Using SSH, Zeng et al., (2006) identified 671 differentially expressed cDNAs in cotton somatic embryos and more than one-third of these cDNAs were not represented in GenBank databases, suggesting SSH is powerful in discovering less strongly expressed genes. In this study, we utilized combination of SSH, microarrays and real time RT-PCR to identify 20 genes differentially expressed in Giza 88 cotton 15-dpa fiber and 5 (13.9%) of them were novel genes. Therefore, combination of SSH, microarrays and real time RT-PCR was an efficient and effective approach for the identification of differentially expressed genes.

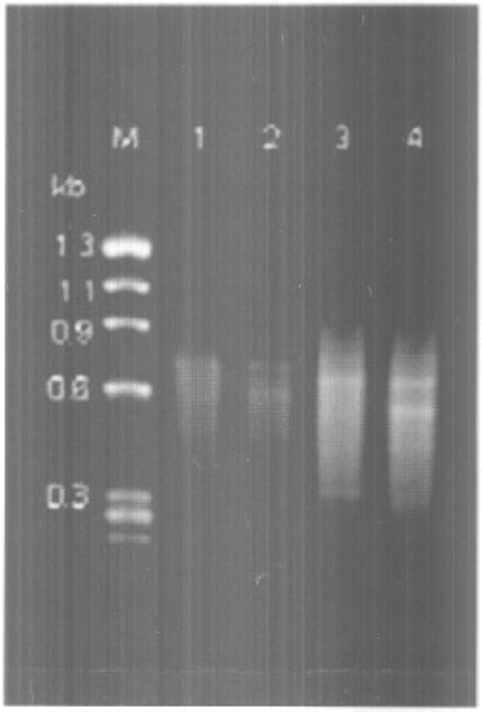

Fig. (1): Analysis of the SSH fragments after the first PCR (Lane 1 & 2) and second PCR (Lane 3 & 4) amplification. M, marker Lane 1 & 3, subtractive tester; 2 & 4, unsubtracted tester.

Table (1): List of differentially expressed genes in Giza 88 fiber relative to Giza 90 fiber.

Clone name	GenBank Accession No.	Annotation	e-value	Functional category	Microarray data Mean of log2 Ratios (Giza88/Giza90)	Real time PCR data Mean of Ratios (Giza88/Giza90)
GZ88-B2	EF464665	AAN32988| beta-tubulin 1	1e-92	Cytoskeleton	1.46	3.46
GZ88-B3	EL737995	EAZ37712| hypothetical protein	1e-13	Unclassified	1.58	23..27
GZ88-B4	EL737996	NP_176628| ubiquitin family protein	1e-21	Protein metabolism	1.23	17.98
GZ88-B5	EL737997	No significant similarity	0.0	Unclassified	1.67	3.41
GZ88-B6	EF464666	NP_568619| ubiquitin conjugating enzyme	2e-76	Protein metabolism	1.14	17.80
GZ88-B7	EL737998	AAQ84323| fiber protein Fb31	5e-45	Unclassified	2.16	7.38
GZ88-B8	EL737999	ABO82378| ubiquitin interacting motif	4e-54	Unclassified	1.37	6.75
GZ88-B9	EL738000	Q39817| calnexin homology precursor	7e-52	Protein metabolism	1.33	4.05
GZ88-B10	EF464667	AAB42144| acetyl-CoA carboxylase	3e-79	Lipid metabolism	1.64	1.85
GZ88-B11	EL738001	NP_567982| unknown protein	1e-20	Unclassified	2.27	13.54
GZ88-B12	EL738002	NP_177929| glycosyl hydrolase family 3protein	7e-32	Energy/carbohydrate metabolism	1.29	6.48
GZ88-B13	EL738003	AAM63491| putative kinesin light chain	2e-56	Cellular structure and organization	2.37	2.66
GZ88-B14	EL738004	BAF02085| hypothetical protein	1e-29	Unclassified	1.36	7.62
GZ88-B15	EF464668	CAM58986| beta-tubulin	2e-61	Cytoskeleton	1.21	1.72
GZ88-B16	EL738005	ABE81294| protein kinase	3e-33	Signal transduction	2.40	3.62
GZ88-B17	EL738006	CAC12882| O-linked GlcNAc transferase	3e-34	Protein metabolism	1.26	3.16
GZ88-B18	EL738007	CAJ77501|expp1 protein precursor	2e-70	Unclassified	1.35	8.14
GZ88-B20	EF464669	BAC23043| pyruvate decarboxylase	2e-108	Energy/carbohydrate metabolism	1.57	22.32
GZ88-B22	EF46467	CAA10285| protein phosphatase	8e-128 8	Protein metabolism	1.39	14.4
GZ88-B23	EF464671	CAC84111| annexin	8e-65	Transport	1.27	13.32
GZ88-B24	EL738010	ABO83276| glycoside hydrolase family 5	6e-70	Energy/carbohydrate metabolism	1.63	20.23
GZ88-B25	EL738011	No significant similarities	0.0	Unclassified	1.38	3.27
GZ88-B26	EL738012	No significant similarities	0.0	Unclassified	1.44	4.74
GZ88-B27	EL738013	ABN08081| unknown protein	3e-25	Unclassified	1.24	2.59
GZ88-B28	EL738014	No significant similarities	0.0	Unclassified	1.23	10.4
GZ88-B29	EF464672	NP_180832| 26S proteasome regulatory subunit	5e-120	Protein metabolism	2.16	19.83
GZ88-B30	EL738015	NP_174029| glycine-rich protein	8e-36	Unclassified	1.49	2.56
GZ88-B31	EL738016	AAU04752| DRP, dynamin-like protein	1e-55	Transport	1.44	2.56
GZ88-B32	EF464673	P93584| actin-82	4e-69	Cytoskeleton	1.52	1.19
GZ88-B33	EL738017	NP_973620| RSZ33 (arginie/serine-rich zinc knuckle-containing protein 33)	5e-11	Nucleic acid metabolism	2.63	3.44

GZ88-B34	EL738018	ABG66307l CCR4 assocaited factor 1-related protein	2e-56	Unclassified	1.37	4.32
GZ88-B35	EL738019	No significant similarities	0.0	Unclassified	1.39	2.43
GZ88-B36	EL738020	AAB86938l NO_ protein	9e-27	Unclassified	1.22	2.4 1
GZ88-B37	EF464674	ABF48494l GDP-mannose pyrophosphorylase	6e-152	Energy/carbohydrate metabolism	1.48	1.59
GZ88-B38	EL738021	ABI34274l IS10 transposase, putative	0.0	Nucleic acid metabolism	1.38	3.01
GZ88-B39	EF464675	AAB37766l cellulose synthase	0.0	Cellular structure and organization	1.65	2.47

Genes related to cytoskeleton, cellular structure and organization

The most highly expressed genes in developing fibers belong to the cytoskeleton gene family, which is responsible for directing polar elongation and thus, contributes significantly to fiber shape. Significant numbers of cell wall and cell wall-related genes are also highly expressed during fiber expansion (Wilkins *et al.*, 2005). In this study, several genes involved in cytoskeleton, cellular structure and organization were identified to be differentially expressed in Giza 88 15-dpa cotton fiber compared to Giza 90 fiber. These genes are two beta-tubulins (EF464665 and EF464668), an actin (EF464673), a putative kinesin light chain (EL738003) and a cellulose synthase (EF464675) (Table 1).

Kloth, (1989) found that the level of tubulin protein increased approximately three fold in cotton fiber from 10 to 20 dpa, and reached a plateau or decreased slightly after 20 dpa, suggesting that the rapid rise in tubulin is correlated with the elongation of fiber and an increase in cellulose synthesis. Two alpha-tubulin and two betatublulin isotypes showed preferential accumulation in 10- and 20-dpa fibers, respectively and this accumulation may be correlated with the dramatic changes in cortical microtubule arrays during cotton fiber development (Dixon *et al.* 1994). One gene encoding beta-tubulin was isolated and characterized, which was differentially expressed in cotton fiber (Li *et al.*, 2002). In the present study authors identified two betatubulin genes differentially expressed in Giza 88 15-dpa fiber compared to Giza 90. Tubulins are major component of micro-tubules. Microtubules play important roles in many basic cellular processes and in higher plant morphogenesis (Kopczak *et al.*, 1992). Cortical microtubules are considered to provide spatial information to the organization of cellulose microfibrils in plant cells (Whittaker and Triplett, 1999). Highly organized microfibrils of cellulose restrict turgor-driven cell expansion to a single major axis of growth during cell elongation (Giddings and Staehelin, 1991; Delmer and Amor, 1995). Therefore, microtubulin arrays play a vital role in cell expansion by controlling the orientation of newly synthesized cellulose microfibrils (Reddy and Day, 2000). One of the differentially expressed genes we identified in Giza 88 fiber was the actin gene. Actin cytoskeleton has a central function in plant cell morphogenesis. The actin cytoskeleton is necessary for cell elongation. Disruption of the actin cytoskeleton by actin antagonists produced distorted trichome with unexpected branches during *Arabidopsis* trichome development (Mathur *et al.*, 1999, Momtaz et al., 2007a&b). RNA interference of cotton actin gene, *GhACT1*, disrupted actin cytoskeleton network in fibers and such action resulted inhibition in fiber elongation (Li et al., 2005). Actin microfilament sometimes co-localize with cortical microtubules in cotton fibers (Andersland *et al.*, 1998). The

organization of cortical microtubules and some of the actin microfilaments parallels the orientation of cellulose microfibrils in cultured fiber cells (Seagull 1990). The interaction between microtubules and actin microfilaments could be mediated by proteins that interact with both cytoskeletal element or by connected proteins that each interacts with one element (Preuss *et al.*, 2004). A cotton fiber kinesin may function on coordinating the interaction between actin microfilaments and microtubules during fiber growth and development (Preuss *et al.*, 2004).

Cotton fiber consists mainly of cellulose which forms more than 90% of the harvested lint fiber. Cellulose synthase plays a vital role on cellulose synthesis. An important breakthrough in understanding cotton fiber and plant cell wall synthesis was the identification of the genes encoding cellulose synthase in cotton fiber using genomic methods (Pear *et al.*, 1996). By comparing to the sequences of bacterial cellulose synthases, Pear *et al.*, (1996) first identified two cellulose synthase (*CesA*) genes expressed at high levels during active secondary wall cellulose synthesis in developing cotton fiber. In this study, we found that a cellulose synthase gene was differentially expressed in Giza 88 15-dpa cotton fiber by about 3-fold increase relatively toGiza 90 cotton fiber (Table 1). Holland *et al.* (2000) identified nine distinct *CesA* genes from maize. By analyzing six of the nine genes using RT-PCR, they found that all six genes were expressed in all the organs examined, and a few of genes selected from maize and Arabidopsis were sound to be expressed in unique cell types engaged in either primary or secondary wall synthesis. Evidence showed that at least some of the *CesA* genes in plants encode a glycosyltransferase and thus, are involved in the process of cellulose synthase (Delmer, 1999). Mutation of the *AtCesA-1* (*Rsw1*) gene in Arabidopsis resulted in significant decrease in cellulose deposition in young cells of seedlings (Arioli *et al.*, 1998), and mutation of *AtCesA-7* (*Irx3*) caused collapsed xylem vessels and reduction of cellulose content in inflorescence stem (Turner and Somerville, 1997; and Taylor *et al.*, 1999). Virus-induced silencing of a cellulose synthase gene in *Nicotiana benthamiana* led to abnormal phenotype of shorter internode length and small leaves and reduced cellulose content in cell wall (Burton *et al.* 2000). These results strongly support that cellulose synthase genes are critical in cellulose synthesis. It has been shown that six of the ten different members of the *CesA* gene family in *Arabidopsis* have non-redundant roles both within and between cell types. *AtCesA1*, *AtCesA3* and *AtCesA6* are all required for primary wall cellulose biogenesis, whereas *AtCesA4*, *AtCesA7* and *AtCesA8* are all required for secondary wall cellulose biogenesis in xylem cells (Haigler *et al.*, 2005).

Genes involved in carbohydrate metabolism

Few genes associated with carbohydrate metabolism were differentially expressed in Giza 88 fiber. These genes include a glycosyl hydrolase family protein (EL738002), a glycoside hydrolase family 5 (EL738010) and a GDP-mannose pyrophosphorylase (EF464674) (Table 1). The synthesis, modification and breakdown of carbohydrate is one of the most fundamentally important reactions in nature. In plants, these processes are of particular importance for cell-wall synthesis and expansion, starch metabolism, defense against pathogens, symbiosis and signaling (Henrissat *et al.*, 2001). Cell expansion is crucial for plant growth and development, and for cotton fiber qualities such as fiber length and fineness. Plant cell expansion occurs through the interaction of varied influences such as cell wall-yield

properties, the opposing force of turgor pressure, the biosynthesis of membrane lipids, new cell wall components and proteins, and the action of wall loosening agents (Cosgrove, 2000; Smart *et al.*, 1999). The latter identified several genes involved in turgor-driven cell expansion of developing cotton fibers, such as genes encoding proton-translocating ATPase, vacuole-ATPase, proton-translocating pyrophosphatase (PPase), phospho*eno*-*l*pyruvate carboxylase (PEPCase). All of these genes but PPase were expressed with the highest level during the period of peak expansion (12-15 dpa), then declined with the onset of secondary cell wall synthesis (Smart *et al.*, 1999). Genes encoding expansin and endo-1,4-_-glucanase (EG) were expressed at the highest level at 9 and 15 dpa fiber (Shimizu *et al*, 1997). Ji et al. (2003) found that the mRNA level of wall-loosening enzyme genes, xylogucan endotransgly-coselase (XET) and expansin, were about 100-fold higher in 10-dpa fiber cells compared to those in 0 dpa ovules, corresponding to their proposed role in cell enlargement. Expansin, XET and EG act as wall loosening agents by loosening or cleaving bonds in the cell wall to allow cell expansion (Cosgrove 2000).

Our findings in this study that the genes encoding a glycosyl hydrolases and a glycoside hydrolase were differentially expressed in Giza 88 15-dpa fiber compared to Giza 90, suggesting that these genes might be involved in cell expansion. Glycosyl hydrolases are a widespread group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non carbohydrate moiety (Henrissat *et al.*, 1995). Hydrolysis of glycosidic bonds is therefore crucial for energy uptake, cell wall expansion and degradation, turnover of signaling molecules (Davies and Henrissat, 1995). Glycoside hydrolase family 5 is one of the largest glycoside hydrolase families, which contains primarily endo-acting enzymes that hydrolyze beta-mannans and beta-glucans (Dias *et al.*, 2004). In plants, the enzymatic hydrolysis of glycosidic bond is particularly important for cell-wall synthesis and expansion, starch metabolism, defense against pathogens, symbiosis and signaling (Henrissat *et al.*, 2001).

One of the genes identified in this study was GDP-mannose pyrophosphorylase (GMPase). GMPase catalyses the synthesis of GDP-D-mannose and represents the first committed step in the formation of all guanosin-391 containing sugar nucleotides found in plants which are precursors for cell wall biosynthesis and the synthesis of ascorbate (Keller *et al.*, 1999). Guanosine-containing sugar nucleotides are formed by a set of reactions in which GDP-D-mannose is first synthesized from D-mannose-1-P by the action of GMPase and subsequently converted to other GDP-sugars. Antisense inhibition of the GMPase led to a significant reduction of the mannose content in leaf cell walls, indicaing that GDP-D-mannose is the sole precursor for the incorporation of mannose into hemicellulose (Keller *et al.*, 1999).

Genes associated with transport

An annexin (EL738017) and a dynamin-like protein (EL738016) genes functioning on transport were identified in Giza 88 fiber. Annexins constitute a class of Ca^{2+}- binding proteins that interact with phospholipid membranes in a Ca^{2+}-dependent manner (Burgoyne and Geisow, 1989). In plants the first annexin-like proteins were identified from tomato (Boustead *et al.* 1989). Andrawis *et al.*, (1993) first identified annexins in cotton fiber. They suggested that these proteins might function on the inhibition of beta-glucan (callose) synthase activity. Shin and Brown, Jr. (1999) reported that the annexin gene was highly expressed in the elongation stages of

cotton fiber differentiation, suggesting a role of this annexin in cell elongation. Annexins have diverse biological functions related to their Ca^{2+}- and phospholipid-binding properties, and are almost certainly involved in mediating some events in calcium-dependent regulation (Andrawis *et al.* 1993; Shin and Brown, Jr., 1999).

Dynamin is one of the group members of high molecular weight proteins with GTPase activity (Obar *et al.*, 1990). Dynamin and dynamin-related proteins are large GTP binding proteins that are involved in membrane trafficking (Kang *et al.*, 2003). In the present study authors reported the identification of a dynamin-like protein in cotton fiber. Gu and Verma, (1996) identified a dynamin-like protein from soybean and this protein may be associated with exocytic vesicles that are depositing cell plate materials during cytokinesis. A plant dynamin-like protein, ADL6, is involved in vesicle formation for vacuolar trafficking at the trans-Golgi network but not for trafficking to the plasma membrane in plant cell (Jin *et al.*, 2001). Kang *et al.*, (2003) identified two members of the *Arabidopsis* dynamin-related protein gene family, ADL1A and ADL1E, which are essential for polar cell expansion and cell plate biogenesis.

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الملخص العربي

تعريف الجينات المرتبطة بنمو تيلة القطن المصرى باستخدام تكنولوجيات SSH, microarays and Real Time RT-PCR.

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** قسم علوم المصادر الطبيعية والبيئية – جامعة الاباما – الولايات المتحدة الامريكية

أحد العوامل المثبطة لتطبيقات الهندسة الوراثية و طرق التهجين التقليدية لتحسين تيلة القطن تتركز فى ندرة المعلومات المتوفرة عن الجينات التى لها علاقة بالتيلة. يوفر القطن المصرى الذى يحمل خصائص التيلة فائقة الطول(الصنف جيزة ٨٨) فرصا نادرة لدراسة الجينات المرتبطة بهذه التيلة وذلك لخصائص التيلة المتميزة بهذا الصنف بالمقارنة بالصنف المصرى جيزة ٩٠ طويل التيلة. ولفهم الاساس الجزيئى لنمو تيلة القطن استخدمنا مجموعة تكنولوجيات ممثلة فى Suppressive subtractive hybridization (SSH) , microarray, and real time RT- PCR وذلك لتعريف الجينات الفعالة والتى لها علاقة بنمو تيلة القطن المصرى. لقد تم استخدام mRNA's معزولة من بويضات زهرة القطن بعد ١٥ يوما من التلقيح وتم عمل واختبار مكتبة SSH cDNA من صنف جيزة ٨٨ المتميز بالتيلة فائقة الطول كمختبر بالمقارنة بالصنف جيزة ٩٠ المتميز بطول التيلة كقاعدة. وقد تم استخدام المكتبات الجينية SSH cDNA's لاختبارها باستخدام ال microarrays . وأدت هذة الاختبارات الى تعريف ٢٠ جين تم التفرقة بينهم ومعبر عنهم فى صنف جيزة ٨٨. وقد تم تأكيد هذة النتائج باستخدام تكنولوجية real time RT-PCR . تحتوى هذة الجينات على ٢ بيتا تيوبيولين – اكتين – كينيزين – سيليولوز سينستاز – عائلة بروتينات الجليكوزيل هيدرولاز- بيروفات ديكاربوكسلاز – عائلة جليكوزيد هيدرولاز – مانوز بيروفوسفوريلاز – بروتين شبيه الدينامين – انيكسين وعدد من الجينات المرتبطة بعملية نقل الاشارة Signal transduction و أيض البروتينات والمادة النووية والدهون.