TITLE: **1182 New Controls of Cotton Fiber Development and Quality Illuminated Through Integration of Genomic, Cell Biological, and Biochemical Analyses**

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ABBREVIATIONS: cDNA, complementary DNA; CoMPP, comprehensive microarray polymer profiling; DPA, days post anthesis; EST, expressed sequence tag; G.h.fbr-sw, for *Gossypium hirsutum* fiber secondary wall, the name for a set of ESTs from 20 DPA fiber; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; RNA, ribonucleic acid

New Controls of Cotton Fiber Development and Quality Illuminated Through Integration of Genomic, Cell Biological, and Biochemical Analyses

ABSTRACT

To better understand cotton fiber secondary wall deposition, a spotted array of 3,185 cDNAs biased toward that stage was probed with RNA from 6, 10, 20, and 24 DPA fiber (*G. hirsutum*). Statistical analysis revealed 276 cotton unigenes that were up-regulated at the transition stage between primary and secondary wall deposition ($p \leq 0.05$ for replicates). Here we describe coordinated analyses undertaken to confer biological meaning to some of these results, including verification of gene expression patterns by use of quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), activity assays for cell wall modifying enzymes, cryo field emission scanning electron microscopy, labeling of particular fiber cell wall epitopes prior to transmission electron microscopy, fiber cell wall glycan epitope profiling and sugar analysis, and manipulation of cotton tissue cultures, all over a time-course of fiber development. These experiments revealed new mechanisms regulating several aspects of fiber development and quality.

KEY WORDS:

cell wall analysis, cotton fiber, elongation, functional genomics, gene expression profiling, secondary wall thickening

This research was aimed at assigning function to genes that are up-regulated for secondary wall deposition in *Gossypium hirsutum* cv. DeltaPine 90. This cultivar was studied because it was the origin of a unique set of expressed sequence tags (ESTs) derived from isolated fiber at the transition between primary and secondary wall deposition (called *Gossypium hirsutum* fiber secondary wall, or G.h.fbr-sw, sequences) (Haigler et al., 2005). It is well known that the secondary wall of the cotton fiber is a critical determinant of yield, single fiber strength, water absorption, and dyeing intensity, but this developmental stage has so far been studied less extensively than fiber initiation and elongation. Through use of diverse technical approaches, we discovered unappreciated cellular aspects of cotton fiber development. Despite their obscurity heretofore, these new developmental mechanisms have substantial implications for control of cotton fiber differentiation and quality. The new findings also draw together and confer larger meaning to observations that have been made before, but not connected into a conceptual whole.

A previous example of the value of a wholistic approach in cotton fiber functional genomics is found in research on sucrose synthase that revealed its roles, through degradation of sucrose, in: (a) generating fructose to support the high turgor required to drive fiber elongation; and (b) supplying UDP-Glc to cellulose synthase enzymes during fiber thickening (reviewed in Haigler et al., 2001; Ruan, 2007). The expression of the gene for this sucrose-cleaving enzyme is simply the springboard for mechanistic control in terms of complex cellular events, which have been partly revealed only through integration of diverse research approaches. For example, elucidating the role of sucrose synthase in fiber development has required use of radioactive and fluorescent tracers in living plant/cell systems, cellular fractionation, protein analysis, state-of-the-art light and electron microscopy, and production of transgenic plants, as well as analysis of gene expression.

The research to be reported here reflects similar work to begin to assign function to other proteins encoded by the genes that are up-regulated at the transition to secondary wall deposition. Surprisingly, we discovered that some of these genes relate to the modification of previously unknown phenomena in the primary cell wall that help to control fiber elongation and likely having implications for secondary-wall-related processes.

MATERIALS AND METHODS

Based on the G.h.fbr-sw ESTs that were biased toward the secondary wall stage of fiber development (Haigler et al., 2005), a microarray of 3,185 cDNAs was prepared. This was hybridized with Cy3/Cy5 probes prepared from RNA isolated from fiber at 6, 10, 20, and 24 DPA. Under the relatively cool growing conditions (26 °C day/ 22 °C night) for the cotton from which fiber was harvested, 20 DPA was early in the transition between primary and secondary wall deposition. The common reference design for the microarray experiment revealed changes in gene expression relative to 20 DPA. Two copies of each amplified cDNA were spotted on each slide and three biological replications and two technical replications (dye swaps) were performed for each data point, followed by quality control then statistical analysis of the results (R Development Core Team, 2005; Smyth 2004, 2005; Smyth and Speed, 2003; Smyth et al., 2005). Briefly, RNA was labeled with the amino-allyl procedure (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml). Hybridization was performed using SlideHyb #1 buffer (Ambion). Slides were scanned using an Affymetrix 428 ArrayScanner and analyzed using the GenePix Pro 3.0 software (Axon). Array normalization and statistical analysis were performed using the "limma: Linear Models for Microarray Data" library module (version 2.2.0) of the R statistical package (version 2.2.0). Slide intensity data were normalized using the

global loess method. The least squares method was used for the linear model fit utilizing the Benjamini and Hochberg method to control the false discovery rate.

Other techniques used to understand the biological significance of some of the up-regulated genes included: (1) precise analysis of fiber developmental stages using the light microscope; (2) cryo field emission scanning electron microscopy of developing cotton fibers observed *in situ* within the boll; (3) observation in the transmission electron microscopic of fiber cell wall molecules labeled with epitope-specific probes (http://cell.ccrc.uga.edu/~mao/wallmab/Antibodies/antib.htm); (4) traditional light microscopic histology; (5) comprehensive microarray polymer profiling (CoMPP) of relative levels of chemical epitopes within extractable polymers of fiber walls (Moller, 2007); (6) analysis of fiber cell wall sugar composition (Zablackis et al., 1995); (7) quantitative real-time reverse-transcription PCR (qRT-PCR) analysis of expression of genes encoding cell wall modifying enzymes in transition- and secondary-wall-stage cotton fiber; (8) assays to prove that enzyme activity increased in correspondence with increases in gene transcript level; and (9) analysis of fiber/ovule tissue cultures.

RESULTS AND DISCUSSION

Statistical analysis of the microarray data revealed 276 cotton unigenes that were up-regulated at 20 DPA (onset of secondary wall deposition) compared to 6 and 10 DPA (elongation via primary wall synthesis). Statistical significance was attributed to changes with $p \leq 0.05$ after: (a) average values were determined from three biological and two technical replications of hybridization, all of which passed quality control; and (b) values from duplicate spots of each cDNA on each slide were combined. The up-regulated cotton unigenes, when translated, were homologous ($E \leq -10$) to 195 unique plant proteins in GenBank, and 3 of these were known only

from cotton. Thirty-nine additional up-regulated G.h.fbr-sw unigenes did not matching any known protein.

Analysis of the homologous proteins in other plants showed that many genes required for secondary wall cellulose synthesis in xylem were up-regulated at the onset of secondary wall deposition in cotton fiber (Table 1; see more complete discussion in Haigler et al., accepted for publication). As examples, genes encoding three secondary-wall-specific cellulose synthase isoforms, a sucrose synthase, a cellulase (KORRIGAN), and a chitinase-like protein were strongly up-regulated at 20 DPA (near the beginning of secondary wall synthesis) compared to 6 and 10 DPA (elongation *via* primary wall synthesis). Also, genes encoding enzymes that are apparently active (by homology to known proteins) in pectin degradation, a pectin esterase and a polygalacturonase, were up-regulated (Table 1). Pectin is found in the matrix of plant primary cell walls, but it is not known to be a component of secondary walls. Pectin esterases (EC 3.1.1.11) remove methyl groups from pectin, which in turn makes the polysaccharide backbone more accessible to random hydrolysis of 1,4-alpha-$_D$-galactosyluronic linkages by polygalacturonases (EC 3.2.1.15). Similar observations on gene expression have recently been discussed in terms of sugar recycling (Guo et al., 2007). Possibly consistent with removal of pectin from the cell wall, arabinose and galactose as well as total uronic acids become abruptly less detectable in cotton fiber wall extracts at the onset of secondary wall deposition (Meinert and Delmer, 1977; Tokumoto et al., 2002).

In these past studies, only extracted polymers were chemically analyzed, so the results could have been misinterpreted if existing polymers became chemically modified and immobilized in cell walls. Therefore, there was previously no unequivocal evidence for primary cell wall modification prior to cotton fiber secondary wall deposition. Also, if such

modifications occurred, there was previously no understanding of their mechanistic implications. Therefore, we investigated further. We confirmed the up-regulation of the cotton genes encoding pectin-degrading enzymes by qRT-PCR and showed that relevant enzymes activities, e.g. pectin methylesterase, were present and developmentally regulated in the same pattern in cotton fiber. Similar analyses by qRT-PCR of more than thirty genes for other cell wall modifying enzymes showed that a subset of them, encoding diverse hydrolases, was up-regulated and had peak expression during the primary- to secondary wall transition. This pattern was distinct from secondary wall cellulose-synthesis-associated genes like *GhCESA1*, *GhCTL1/2*, and the cotton *KORRIGAN* homologue. These three genes also up-regulated at the transition stage, but they had sustained high expression during secondary wall deposition.

To understand the nature of the cell wall modification, we carried out CoMPP profiling of cotton fiber cell wall glycan polymers that were sequentially extracted in three solvents: (1) 50 mM diamino-cyclo-hexane-tetra-acetic acid (CDTA), pH 7.5; (2) 4 M NaOH with 1% (v/v) $NaBH_4$; then (3) cadoxen (31% v/v 1,2-diaminoethane with 0.78 M CdO). Typically, these solubilize pectins, other non-cellulosic wall matrix components, and cellulose, respectively (Moller et al., 2007). Extractions were made of fiber between 6 – 30 DPA with special emphasis on days during the transition between primary and secondary wall deposition. Fiber extracts spotted onto membranes in a microarray were probed with monoclonal antibodies or carbohydrate binding modules that bound to cell wall polymers, then signals were analyzed similarly to a nucleic acid microarray. These results showed that particular cell wall epitopes were diminished at the transition stage. In an extension of previous results (Meinert and Delmer, 1977; Tokumoto et al., 2002), analyses of sugars in cell wall polymers also showed loss of particular sugars, but not others, at the transition stage.

The cellular nature and significance of the cell wall modification was revealed through electron microscopy: (a) transmission electron microscopy including labeling of particular cell wall components; and (b) cryo field emission scanning electron microscopy to view fibers within nearly intact cotton bolls in their "as living" condition. These images revealed new features of the cotton fiber primary wall that affected elongation, as well as showing how the primary wall was modified at the onset of secondary wall deposition. Traditional methods of light microscopic histology, as well as cryo field emission scanning electron microscopy, revealed the implications of these images at a larger scale.

The summation of the data allowed us to: (a) propose a consequence of the spiral growth of young fibers that was previously observed (Stewart, 1975) and confirmed by us; (b) understand why rather organized groups of primary-walled fibers have sometimes been seen (Goynes et al., 1995); (c) understand the origin of wave-like patterns of small groups of fibers (Xie et al., 1993: Paiziev and Krakhmalev, 2004); (d) determine how ~500,000 fibers are able to undergo extreme elongation within the confined space of the cotton boll; (e) interpret the report that fiber diameter expands at the onset of secondary wall deposition (Seagull et al., 2000); and (f) see additional constraints on fiber uniformity. Further implications of the new developmental mechanisms are currently being explored through manipulation of cotton ovule/fiber cultures. Clearly, multiple technical approaches are required to understand the functional consequences for expression of particular genes at the different stages of cotton fiber development.

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Table 1. Genes up-regulated at the primary-to-secondary wall transition in cotton fiber as determined by spotted cDNA microarray analysis. In all cases, up-regulation at 20 DPA compared to 10 DPA was significant at $p \leq 0.004$.

Gene Name or Type	GenBank Accession ID for the EST on the array	20/10 DPA expression ratio, $\log_2$
GhCESA1 **(*AtCESA8*-like)**	**CO498265**	**4.8**
GhCESA2 **(*AtCESA4*-like)**	**CO491935**	**2.7**
***AtCESA7*-like**	**CO495561**	**4.2**
Sucrose synthase	**CO497506**	**1.3**
***KORRIGAN*-like**	**CO492974**	**1.4**
GhCTL1/2	**CO493183**	**6.4**
Pectin methylesterase	**CO495780**	**2.2**
Polygalacturonase	**CO492632**	**2.5**