Chapter 10

RECENT ADVANCES IN COTTON FIBER DEVELOPMENT

Michael R. Stiff1 and Candace H. Haigler2
Department of Crop Science1 and Department of Plant Biology2
North Carolina State University
Raleigh, NC 27695

INTRODUCTION

Cotton fiber is the world’s most important natural textile fiber. In the U.S.A. in 2010, cotton fiber had a 61% share of the market for apparel and home textiles (Bearden, 2010), with synthetic fibers having most of the remaining market share. A similar demand for renewable cotton fiber occurs worldwide. A highly regulated cellular differentiation process governs the morphogenesis of the fiber. Each long cotton “lint” fiber originates from a single epidermal cell on the ovule surface that transforms into the highly elongated and reinforced dead fiber through dramatic polar expansion and cell wall thickening. Fiber morphogenesis proceeds through several stages that will be described further in this chapter: initiation, elongation, transitional primary wall remodeling, secondary wall synthesis, and maturation. These differentiation processes, which typically last at least 50 days, directly determine cotton fiber quality characteristics. The number of fibers initiated in the outer integument of the ovule is a major factor in fiber yield. Fiber fineness (weight per unit length) and micronaire are determined by the fiber perimeter and the extent of secondary cell wall thickening. Fiber length and length uniformity are both valued in modern spinning mills, and cell elongation is strongly affected by developing and maintaining high turgor pressure within the central vacuole as well as carbon supply in different regions of the seed. Fiber strength is affected by properties of the transitional “winding” cell wall layer as well as secondary wall cellulose (e.g., degree of polymerization and microfibril angle). High fiber tensile properties (including strength and mechanical elongation, or elongation-to-break) help to preserve fiber length during processing, and they are also required to produce strong yarns and fabrics. The potential of cotton fibers to pickup dye molecules and to absorb water are determined by the amount and the degree of crystallinity of the secondary wall cellulose. The final collapse of the fiber into the typical kidney bean shape that facilitates spinning (through improved friction properties) relies on adequate filling, but not over-filling, with secondary wall cellulose (Wakelyn et al., 2007). Since a cotton fiber is a cell wall composite, many of its important developmental processes relate to plant cell wall deposition (Haigler et al., 2012).

At first, it was surprising that the single-celled cotton fiber expresses a large percentage of the genes present in a complex allotetraploid genome (Hovav et al., 2008b), but the morphogenetic processes leading to commercial cotton fiber encompass many aspects of whole plant growth. Many years will be required to understand how most of the genes and regulatory networks ex-
ert their effects on the fiber differentiation process. Cotton fiber has many advantages for such experiments. It is a single cell that undergoes semi-synchronous differentiation in a series of overlapping developmental stages. Cotton fibers are easily separated from the developing seed. Therefore, researchers can sample a large population of one cell type and have clear knowledge of the predominant cellular activities at that time. Given that cotton fibers are expendable for plant growth, there is unrestricted ability to manipulate fiber development experimentally. The genus *Gossypium* includes living, non-domesticated, diploid and allotetraploid progenitor species that provide valuable comparisons and contrasts with commercial fiber (Kim and Triplett, 2001; Hovav et al., 2008b; Rapp et al., 2010).

Below we provide a review of emerging experimental evidence on the cellular and physiological processes underlying the morphogenesis of domesticated *G. hirsutum* fiber with emphasis on research published since 2005. Useful related information can be found in other reviews discussing: cotton fiber-ovule culture as a unique experimental tool (Kim and Triplett, 2001); the evolutionary and domestication history of cotton (Wendel et al., 2009); genomic scale research on the secondary wall thickening phase (Haigler et al., 2005; Haigler et al., 2009); transcriptional changes during fiber development (Wilkins and Arpat, 2005; Shangguan et al., 2010); and transcriptional and hormonal regulation of fiber development (Lee et al., 2007).

**COTTON FIBER DEVELOPMENT**

**Cotton Fiber Initiation**

The first step in cotton fiber morphogenesis is the differentiation of selected ovule epidermal cells into fiber initials, or rounded protrusions above the ovule surface. Fiber initiation typically begins on the day of anthesis and continues to at least 5 days post anthesis (DPA) when each ovule has about 16,000 fiber initials (inclusive of young elongating fibers) in modern cultivars (Fig. 1; Stewart, 1975; Seagull and Giavalis, 2004). As initials expand, the central vacuole forms and the nucleus migrates from the base toward the middle of the developing fiber. The density of fiber initials at 0 and 1 DPA was positively correlated with lint percentage and lint index in five *G. hirsutum* cultivars (Li et al., 2009). Humans selected for higher density and more synchronous fiber initiation during cotton domestication (Butterworth et al., 2009). Generation of turgor pressure within the central vacuole drives the expansion of fiber initials. Ruan and coworkers (Ruan et al., 2003) showed that both fiber initiation and fiber elongation were inhibited by suppressed expression of the SS3 isoform of sucrose synthase (Sus) in transgenic cotton (Ruan et al., 2003). Plants with lower Sus protein in the seed coat compared to wild-type cotton produced fewer fibers that appeared shrunken and collapsed when viewed by scanning electron microscopy. The transgenic fibers had lower Sus activity, which correlated with decreased levels of hexoses and starch, but not sucrose. Possibly decreased hexoses caused lower osmotic potential and reduced turgor, but the fiber defects might also have been at least partly explained by factors such as less Sus-generated UDP-glucose to provide the substrate for cell wall synthesis (see further discussion below) and/or disruption of signaling pathways that might depend on hexose.
Figure 1. Representation of a mature cotton plant containing bolls and fibers at all stages of development. The stem indicates the fastest timeline for cultivated fiber development when plants are grown in an optimal (30°C) environment. Branches indicate days post-anthesis (DPA) and the images show many of the key features of fiber development. Cryo-field-emission scanning electron microscopy of (A) fiber initials on the ovule surface (bar = 10 µm); (B) twisting and elongating 3 DPA fibers (bar = 100 µm); (C) cotton fiber middle lamella (CFML) stretched between two 3 DPA fibers (bar = 4 µm); (D) ordered bundles of fibers inside the boll (bar = 100 µm). Differential interference contrast micrographs indicating microfibril angle of (E) 16 DPA and (F) 20 DPA fiber, with a steeper angle at 20 DPA (bars = 10 µm). TEM fiber cross-section showing (G) an early stage of secondary wall thickening (bar = 300 nm); and (H) a more advanced stage of secondary wall (bar = 1 µm). (I) Mature cotton boll and (J) cross-section of mature fiber viewed in the light microscope. SCW, secondary cell wall.
Transcriptional Regulation of Initiation

Transcription is regulated by various small RNAs, such as small interfering RNAs (siRNAs), as well as the activity of transcription factors. Small RNAs occur in size classes that regulate gene expression differently. siRNAs are predominantly 24 nucleotides long and direct DNA methylation and chromatin remodeling. The typically 18-21 nucleotide microRNAs (miRNAs) are derived from an endogenous hairpin structure and participate in the RNA-induced silencing complex (RISC) to target homologous mRNA for degradation or inhibit translation (Voinnet 2009). Some of the miRNAs expressed in cotton fiber have been identified by bioinformatics (Zhang et al., 2007) and, along with siRNAs, through sequencing approaches (Pang et al., 2009; Kwak et al., 2009). Ovules engaged in early fiber development (0 and 3 DPA) showed a marked increase in the expression of 24 nucleotide siRNAs compared to -3 DPA ovules and leaves, which led to the proposal that siRNA-mediated chromatin remodeling may contribute to fiber initiation. In general, miRNAs were expressed more highly in -3 DPA ovules compared to 3 DPA fiber-bearing ovules and 10 DPA fibers, suggesting that miRNAs may maintain low expression of target genes prior to initiation (Pang et al., 2009). Consistent with a decline in miRNAs by 3 DPA, a large percentage of the cotton genome is expressed in 2 to 25 DPA fiber (Hovav et al., 2008b). In the *fussless-lintless* (*fl*) mutant that does not initiate fiber, more miRNA families were expressed compared to wild-type, leading to the hypothesis that targeted down-regulation of genes controlling fiber initiation could underpin the mutant phenotype (Kwak et al., 2009).

Transcription factors, often working together in complexes, bind with specific genomic sequences to promote, enhance, or block transcription. Given the importance of fiber initiation for fiber yield, several studies have focused on identifying transcription factors that control initiation. In the regulatory cascade controlling fiber initiation, GhMYB25-like (Walford et al., 2011) acts up-stream of GhMYB25 (Machado et al., 2009). The promoter of *GhMYB25-like* drove GUS expression from -3 DPA to 3 DPA in the ovule surface and elongating fibers, and RNAi suppression of *GhMyb25-like* caused fiberless seeds. However, over-expression of *GhMYB25-like* did not increase the number of fiber initials. Consistent with these data, -1 to 4 DPA ovules of *fl* mutant had reduced *GhMYB25* expression, and only a mutated A genome homolog with a single amino acid substitution in the DNA binding domain was expressed in contrast to expression of wild-type A and D homologs in the parental line (Walford et al., 2011). *GhMYB25* was identified in Southern blots of diploid species *G. arboreum* (A2 genome), *G. raimondii* (D5 genome), and the allotetraploid *G. hirsutum* cv. Coker 315 (AD genome) (Machado et al., 2009). *GhMYB25* was highly expressed in 0 DPA ovules, and its promoter activated the *GUS* reporter in epidermal cells of 0 DPA ovules and 10 DPA fibers. RNAi suppression of *GhMYB25* resulted in 10-20% fewer fiber initials, and the initials that formed had delayed expansion and elongation compared to wild-type. In contrast, over-expression of *GhMYB25* resulted in 15-35% more fiber initials with no alteration of final fiber length, supporting the participation of the GhMYB25 transcription factor in controlling fiber initiation. Notably, as discussed by Walford and coworkers (Walford et al., 2011), GhMYB25-like and GhMYB25 are more similar to petal-expressed MYBs in several species than they are to the MYBs that regulate arabidopsis leaf trichome formation, which correlates with known differences between the pathways regulating formation of cotton fibers and arabidopsis leaf trichomes (Serna and Martin, 2006).
Other initiation-associated transcription factor genes have recently been characterized. The RAD-Like *GbRL1* from *G. barbadense*, a SANT/MYB-type transcription factor, was expressed most highly in cotton ovules at -3 and 0 DPA. Over-expression in arabidopsis caused dwarfining and delayed flowering, similar to the effects of other RAD genes (Zhang et al., 2011a). *GaHOX1* was isolated from *G. arboreum* (Guan et al., 2008) as a homolog of arabidopsis GLA-BRA2 (GL2), a gene required for cell expansion, branching, and cell wall maturation in leaf trichomes. *GaHOX1*, a member of the class IV homeodomain-leucine zipper (HD-ZIP) family of transcription factors, was expressed in many *G. arboreum* tissues but most strongly in 0 DPA ovule epidermal cells and 1 DPA fiber. In *G. hirsutum* the strongest expression was during early fiber elongation. Supporting a possible role in fiber initiation, *GaHOX1* complemented the trichome-less phenotype of the arabidopsis *gl2-2* mutant (Guan et al., 2008). Evidence for a temporal cascade of transcription factors in early fiber development was obtained by comparing gene expression profiles in TM-1 and its derivative lintless mutant, *N1N1*, over the period of -3 to 10 DPA (Lee et al., 2006). Five distinct gene expression profiles were identified, and certain genes were up-regulated in a logical progression (as often inferred from the function of their arabidopsis homolog). For example, *GhPDF1* (protodermal factor 1 that is possibly involved in cell fate determination) was highly expressed in -3 DPA ovules, then *GhMYB25* was expressed at 0 DPA, followed by the up-regulation of other genes such as *E6*, *EF-1*, and *RDL1* by 3 DPA and afterwards as fiber elongation progressed. Guan and coworkers (Guan et al., 2011) suggested a specific pairing of a transcription factor and its downstream target based on co-expressing *GhMYB2* and *GhRDL1* in arabidopsis, which induced ectopic seed and silique trichomes. *GhMYB2* functioned similarly to arabidopsis GL1 (involved in leaf trichome development), and *GhMYB2* targeted the *GhRDL1* promoter. Expressing either gene alone increased the number of arabidopsis seeds producing trichomes (~6%) compared to wild-type (~0.4%). Co-expression led to ~8% or ~10% seed trichomes in wild-type or the *try* mutant background, respectively. The induced seed trichomes were at least two times longer than in wild-type. The results led to a regulatory model involving *GhMYB2*, *GhMYB25*, *GhMYB109*, and *GhTTG1* as controllers of the downstream transcription of *GaHOX1* and *GhRDL1*. The data also predicted the existence of a cotton protein that functions as a negative regulator of fiber initiation, similar to TRIPTYCHON (TRY) in arabidopsis trichomes.

**Action and interaction of phytohormones on fiber initiation**

Phytohormones participate in signaling processes to regulate almost every aspect of plant growth and adaptation. Many years ago, gibberellic acid (GA) and auxin (IAA) were shown to be required for fiber growth on cultured cotton ovules (Beasley and Ting, 1973), and recent large scale gene expression data on -3 to 3 DPA ovules (or ovules with fibers) compared to other cotton tissues implicated extensive networks related to the biosynthesis and signaling of IAA, GA, and brassinosteroids (BR) as important for early fiber development (Yang et al., 2006).

There is experimental evidence for a positive effect of IAA on fiber initiation. Zhang and co-workers (Zhang et al., 2011b) observed IAA accumulation in the outer integument of the ovule at 0 DPA, but not in ovules treated with an IAA transport inhibitor (1-napthalamic acid, NPA) or
in the fiberless, fl, mutant. Cotton was transformed with an IAA biosynthesis gene, *iaaM*, under the control of the fiber-specific *FBP7* promoter, which resulted in 128% higher IAA concentration at 0 to 3 DPA, 13.5-28.6% more fiber initials, and 19.9-39.8% increase in the number of lint fibers compared to wild-type. The *FBP7* promoter was strongly effective between -2 to 0 DPA, which might reflect the period that IAA acts in the epidermis to stimulate cotton fiber initiation. This hypothesis was supported by unchanged fiber initiation when the *iaaM* coding sequence was over-expressed under the control of the promoter of the *E6* gene, which has maximal expression at 5 to 24 DPA (Zhang et al., 2011b). Ovules from -3 to 3 DPA also expressed homologs of genes from other species that are related to IAA biosynthesis (*YUCCAs*, *CYP83B1s*, *NIT2*) as well as IAA signaling and transport (*ARFs*, *AUX1*, *TIR1*, *PIN1*) (Yang et al., 2006).

GA has a positive effect on both initiation and elongation of the cotton fiber. Addition of GA to the medium of cultured ovules stimulated fiber elongation, whereas addition of the GA biosynthesis inhibitor paclobutrazol resulted in fewer, shorter fibers compared to no hormone controls (Liao et al., 2009). Ovules from -3 to 3 DPA expressed homologs of genes from other species that are involved in GA biosynthesis (*GA20OX*, *GA20OX*, *POTH1*, *KO*) and GA signaling (*GAI*, *RGL2*, *RGL1*, *DDF1*, *PHOR1*, *RSG*, *PKL*, *GL1*, *GAMYB*, *AGAMOUS*, *LUE1*) (Yang et al., 2006). Constitutive over-expression of *GhGA20ox1* in cotton resulted in more fiber initials and elongating fibers at 0 to 3 DPA and significantly higher level of bioactive GA in 0 DPA ovules and 10 DPA fibers (Xiao et al., 2010). The role of GA in fiber elongation will be discussed further below.

Experimental evidence also supports a role for BR in fiber development. Treating cotton flower buds with the BR biosynthesis inhibitor, brassinazole2001, arrested fiber initiation, possibly because inhibition of BR biosynthesis altered epidermal cell differentiation (Sun et al., 2005). The expression of genes known from other species to be involved in BR biosynthesis (*SMT1*, *SMT2*, and *BR60X*) and signaling (*BR1ls*, *BAK1*, *BES1*, *CPD*) was enriched in -3 to 3 DPA ovules compared to elongating fibers (Yang et al., 2006). The cotton gene *GhDET2* is homologous to arabidopsis *AtDET2*, which is responsible for the rate-limiting step in BR biosynthesis. *GhDET2* had highest expression in ovules and elongating fibers, and heterologous expression showed that the encoded protein functioned as expected as a progesterone reductase. When *GhDET2* expression was suppressed in transformed cotton, fewer fiber initials formed and fiber elongation was inhibited. In contrast, increasing *GhDET2* expression in the seed coat led to more, longer fibers (Luo et al., 2007).

**Effect of ROS and Ca2+ on fiber initiation**

Other signaling molecules that have been studied recently for their role in cotton fiber development include calcium (Ca2+) and reactive oxygen species (ROS), specifically superoxide (O2•−) and H2O2. ROS are produced in response to various biotic and abiotic stressors, and high levels cause oxidative stress and often cell damage. ROS are also important signaling molecules during plant development (Mittler et al., 2004; Swanson and Gilroy, 2010). In cotton, ROS were detected in fiber initials at 0 DPA through fluorescence of the ROS indicator 2’, 7’-dichlorodi-hydrofluroscein diacetate (2, 7-DCH2FDA) (Mei et al., 2009). When bolls from *G. hirsutum* fiber initiation mutants, naked seed (*N1*) and fuzzless Xinlianxiaoji (*XinFLM*), were treated
with \( \text{H}_2\text{O}_2 \), fiber initials were induced in both mutants by 0 DPA (Zhang et al., 2010). These experimental data support a role for ROS in fiber initiation.

\( \text{Ca}^{2+} \) is key to many cellular processes (Kudla et al., 2010), and research has begun to describe how \( \text{Ca}^{2+} \) participates in cotton fiber initiation and elongation. \( \text{Ca}^{2+} \) accumulation was correlated with fiber initiation and ER development in 0 DPA ovules compared to -1 DPA ovules. Microarray analysis showed that genes encoding components of \( \text{Ca}^{2+} \) signaling, such as calmodulin binding protein, were up-regulated at 1 DPA (Taliercio and Boykin, 2007).

**Cotton Fiber Elongation**

After initiation, polar expansion and rapid fiber elongation proceed from \( \sim 2 \) to 20 DPA until the fiber is 2 to 3 cm long. Beginning as early as 2 to 3 DPA, elongating fibers twist together to form bundles of fiber (Fig. 1B, C; Singh et al., 2009a; see further discussion below on the cotton fiber middle lamella). The recently characterized cotton vacuolar invertase (GhVIN1) appears to play a role during early elongation by increasing osmotica for the control of turgor. GhVIN1 expression and VIN activity peaked at 0 to 5 DPA in \( G. \text{hirsutum} \) fiber, but VIN activity was higher and persisted at a high level until 10 DPA in \( G. \text{barbadense} \) fiber that was also longer than in \( G. \text{hirsutum} \) (Wang et al., 2010b). Other specific cellular mechanisms facilitate the most rapid phase of cotton fiber elongation. For example, increased synthesis of callose was correlated with closing of plasmodesmata at the fiber foot from 10 to 16 DPA. The isolation of the fiber from the ovule was proposed to result in higher turgor pressure, which in turn facilitated the period of most rapid fiber elongation (Ruan, 2007). Aquaporins, membrane proteins that facilitate the movement of water across biological membranes, may also have a role in cotton fiber elongation. Cotton aquaporins include GhγTIP1 located in the tonoplast and GhPIP1-2 located in the plasma membrane (Liu et al., 2008). Both genes were expressed at 5 to 15 DPA during fiber elongation, and further work is needed to clarify a potential role of aquaporins in regulating turgor-driven elongation. Starch exists in elongating cotton fiber, and at 10 DPA there was peak expression of the genes encoding the subunits of ADP-glucose pyrophosphorylase that are required for starch biosynthesis (Taliercio, 2011). However, starch composed only \( \sim 0.3\% \) of fiber dry weight, which correlates with only rare small starch grains observed at the level of transmission electron microscopy (TEM) in cotton fiber (Haigler and coworkers, unpublished).

**Transcriptional and proteome-level regulation of elongation**

The R2R3 MYB family transcription factor, GhMYB109, is structurally similar to AtGL1 and AtWER, which help to regulate arabidopsis leaf trichome development (Pu et al., 2008). Logically, GhMYB109 acts downstream of initiation-related GhMYB25-like and GhMYB25 (Walford et al., 2011). The GhMYB109 promoter drove fiber-specific gene transcription, and antisense suppression of GhMYB109 led to decreased fiber length. In addition, the decreased expression of GhMYB109 was also associated with the suppression of genes known to be involved in fiber elongation, e.g., GhACO1 and GhACO2 that support ethylene (ET) biosynthesis and GhTUB1 and GhACT1 encoding cytoskeletal proteins. Therefore, GhMYB109 likely acts upstream of phytohormone and cytoskeletal changes during fiber elongation (Pu et al., 2008).
Moving beyond the transcriptome to analyze proteins showed that the cotton fiber proteome is dynamic and temporally regulated. Total protein (% of extracted fiber frozen weight) decreased from 5 to 20 DPA, then again from 30 to 35 DPA during fiber development in *G. hirsutum* cv. CRI 35. A total of 235 proteins showed changing abundance between 5 to 25 DPA in two dimensional gel electrophoresis, and these clustered into four distinct abundance patterns during fiber development (Yang *et al.*, 2008). Possibly helping to control the dynamic fiber proteome through regulating protein degradation, a cotton RING-type ubiquitin ligase (E3), GhRING1, was identified. Transcription of *GhRING1* increased from 5 to 15 DPA then decreased from 15 to 23 DPA, and recombinant GhRING1 had ubiquitin ligase activity *in vitro* (Ho *et al.*, 2010).

**Action and interaction of phytohormones in fiber elongation**

In recent years, the exogenous effect, endogenous amount, and genes involved in the signaling and biosynthesis of phytohormones have been studied in the elongating cotton fiber. Complementing the work of Liao and coworkers already described (Liao *et al.*, 2009), Aleman and coworkers (Aleman *et al.*, 2008) determined the effect of exogenous GA on fiber development while describing components of GA perception in cotton. Adding GA3 to cultured ovules for 10 days stimulated ovule growth, fiber elongation, and expression of elongation-related genes such as *EXP*, *XTH1*, and *XTH2*. In addition, cotton homologs of the rice GA receptor gene *OsGID1*, *GhGID1a* and *GhGID1b*, and cotton DELLA-type genes, *GhSLR1a* and *GhSLR1b*, were identified. DELLA proteins repress GA-mediated gene expression and cell growth and enable fine control of hormone responses. The function of *GhGID1a* as a GA receptor was supported by its ability to rescue rice *gid1-3* mutants. The role of *GhSLR1b* as a DELLA protein was supported by its over-expression in arabidopsis causing a dwarfed phenotype (Aleman *et al.*, 2008). In further analysis of *GhSLR1*-type genes, the expression of a similar gene, called *GhRGL*, increased in ovules/fiber until 10 DPA (Liao *et al.*, 2009). (Note: *GhRGL* was amplified using primers designed from the same full-length cotton nucleotide sequence as one of the two used by Aleman *et al.* (2008) to identify *GhSLR1a* and *GhSLR1b*. *GhRGL* may be an allele of or the same sequence as one of these two genes.) Similar to results for *GhSLR1b* and consistent with function as a DELLA protein, arabidopsis constitutively expressing *GhRGL* was dwarfed, and plant height was negatively correlated with gene expression (Aleman *et al.*, 2008). Study of GA biosynthesis in the fiber has added more to this story. *In vivo* levels of GA in *G. hirsutum* were found by mass spectrometry (LC-ESI-MS) to be highest in fibers at 10 DPA, which was also the time of highest expression of the GA biosynthesis gene, *GhGA20ox1* (Xiao *et al.*, 2010). Over-expression of *GhGA20ox1* in *G. hirsutum* led to significantly longer fibers at 5 DPA and at maturity. The effects of altering GA *in vitro* and *in vivo* on fiber length and the dynamic nature of GA concentration during fiber development demonstrated a role for GA in regulating elongation. Consistently, genes relevant to the biosynthesis, perception, and control of GA demonstrate the phytohormone’s relevance within cotton fiber.

The effect of BR on fiber elongation has been explored by manipulating ovule cultures and characterizing the expression of BR biosynthesis and signaling genes. The most biologically active BR, brassinolide, and the BR biosynthesis inhibitor brassinazole2001 have been used
to examine the effect of BR on fiber length and gene expression (Sun et al., 2005). The addition of brassinolide increased fiber length by 12.7%, whereas inhibition of BR biosynthesis decreased fiber length 38% after 14 days in ovule culture. Further, brassinolide stimulated the expression of the elongation-related genes EXP, XTH, AGP, and GhTUB1, whereas brassinazole2001 inhibited their expression. Fiber elongation was also decreased when finasteride, another BR biosynthesis inhibitor, was added to cultured ovules for 5 or 15 days, but the inhibition was reversed by adding BR to the medium (Luo et al., 2007). The cotton homologs of AtBIN2, encoding a negative regulator of BR signaling that makes plants insensitive to BR have also been investigated (Sun and Allen, 2005). The expression of GhBIN2-C and GhBIN2-E were highest in cotton tissues undergoing rapid cell expansion and/or vascularization, including ovules at 5 to 8 DPA bearing elongating fiber. This work further showed that, as predicted, over-expression of GhBIN2 in arabidopsis led to dwarfism that correlated with the level of transgene expression, i.e., transgenic arabidopsis expressing more GhBIN2 resulted in shorter plants. BR biosynthesis is also active and required for fiber elongation, as already described for GhDET2 (Luo et al., 2007).

Abscisic acid (ABA) is a phytohormone associated with the inhibition of elongation in some plant cells (Lee et al., 1994). The effect of ABA on cotton fiber elongation was examined for three cotton genotypes representing long, medium, and short fibers: G. hirsutum Hybrid-4 and Hybrid-8 as well as G. arboreum Gujarat Cotton-15 (Dasani and Thaker, 2006). In ovule culture experiments, addition of ABA decreased fiber elongation compared to no hormones, and fiber elongation was negatively correlated with increasing ABA concentration when NAA and GA3 were also in the medium. In field-grown fiber, the ABA concentration measured by ELISA increased after fiber elongation ended even though the maximum fiber length occurred at 33 DPA in both G. hirsutum cultivars or 19 DPA in the G. arboreum cultivar. Together the in vitro and in vivo results suggest that ABA may actively inhibit elongation and/or positively signal the beginning of fiber wall thickening.

Ethylene is involved in signaling mechanisms related to fruit ripening, flower development, and stress responses (Bleecker and Kende, 2000). ET has been detected in fibers, it affects fiber elongation, and genes for ET biosynthesis are transcribed during elongation. The level of ET in cultured ovules was significantly decreased by treatment with the ET biosynthesis inhibitor, L-(2-aminoethoxyvinyl)-glycine (AVG). ET promoted fiber length, and AVG inhibited fiber elongation in a concentration dependent manner. Microarray analysis and metabolic mapping of gene expression in developing fiber identified ET biosynthesis as the most up-regulated pathway. PCR confirmed that the ET biosynthesis genes, ACO1 and ACO2, were up-regulated in 5 to 15 DPA fiber, whereas ACO3 peaked earlier at 10 DPA. Yeast expressing each of the three ET biosynthesis genes produced ET, verifying their function in ET biosynthesis (Shi et al., 2006).

ET may work in conjunction with other signaling pathways such as BR and ROS. Treating cultured ovules with ET overcame the hindrance of fiber elongation resulting from inhibition of BR biosynthesis, and ET or BR treatment stimulated the expression of biosynthesis genes of the other hormone. Action within an integrated pathway is supported by no additive effect on fiber length after dual ET and BR treatment (Shi et al., 2006). With regard to ROS, transcription of the cotton ascorbate peroxidase gene, GhAPX1, APX activity, and fiber length were increased
after young fibers in culture were treated with H₂O₂. ET treatment also increased the level of H₂O₂ in fibers (Li et al., 2007). Later work showed that treatment of cultured ovules with H₂O₂ promoted ET production, which led to the proposal that ET stimulates both H₂O₂ production and fiber elongation while H₂O₂ stimulates additional ET synthesis (Qin et al., 2008).

There is evidence that ET responds to an extracellular ATP/ADP signal, with a low dose of extracellular ATP/ADP stimulating fiber elongation whereas a higher concentration is inhibitory. The authors argued that the effects were due to perception of the molecule within a signaling process rather than phosphate transfer (Clark et al., 2009). For example, 30 µM of the ATP-analog, ATPγS, stimulated fiber elongation, whereas 150 µM ATPγS inhibited fiber elongation. Neither effect occurred when ET biosynthesis was inhibited by AVG or AgNO₃. Addition of the ET precursor, ACC, also reduced the concentration of ATPγS required to stimulate elongation from 30 µM to 10 µM. The effects were also observed using poorly hydrolysable ATP- and ADP-analogs. A similar biphasic effect of high and low concentrations of the extracellular nucleotide on arabidopsis root hair showed that ROS and nitric oxide (NO) were required to propagate the effect of the nucleotides (Clark et al., 2010), and similar interactions may take place in elongating fiber. ET in cotton fiber is also influenced by very-long-chain fatty acids (VLCFA) (Qin et al., 2007a), which will be discussed below. Further work to elucidate the connection among extracellular VLCFA, ATP, ET, BR, and ROS could reveal a complex chemical communications network coordinating the elongation stage of fiber development.

ROS occurrence throughout fiber elongation

ROS are important regulators of cotton fiber elongation as shown by fiber transcriptomics and proteomics, manipulation of intracellular ROS in fibers of cultured ovules, and characterization of fiber-specific genes involved in ROS management. The most stable ROS as a transmissible signaling molecule is H₂O₂, which accumulates at low levels during early elongation until it peaks at 20 DPA (Potikha et al., 1999; Yang et al., 2008). Microarray comparison of global gene expression between the long fibered G. herbaceum and the short fibered G. longicalyx revealed genes involved in stress-responses, including oxidative stress, were over-represented during early fiber development in the short fibered species. This led to the hypothesis that the longer fiber in more recently evolved or selected cotton had been supported by more robust systems for ROS management (Hovav et al., 2008a). The expression of three genes involved in ROS management (GAST1-like, Cop1/BONZAI, and Pex1) was higher in long fibered species (G. herbaceum, G. arboreum, G. hirsutum cv. TM1, and even wild G. hirsutum var. yucatanense) compared to short fibered species (G. longicalyx and G. raimondii). Microarray analyses of fiber gene expression in domesticated G. hirsutum and G. barbadense and their wild progenitors also showed that both domesticated species displayed increased expression of antioxidant genes during early elongation (Chaudhary et al., 2009). Changes in the fiber proteome during elongation support these observations. For example, dehydroascorbate reductase involved in redox homeostasis was most abundant at 5 to 15 DPA. The activity of ascorbate peroxidase, which uses ascorbate to reduce two H₂O₂ to two H₂O and O₂, peaked at 10 DPA and subsequently dropped while H₂O₂ concentration then increased (Yang et al., 2008). Another study showed that GhAPX1 transcript levels and APX activity were highest at 5 to 10 DPA, and APX
protein was more abundant in wild-type cotton fiber compared to the *fuzzless-lintless fl* mutant (Li *et al.*, 2007). Cu/Zn-superoxide dismutase (SOD) genes have also been identified in cotton. Cytosolic GhCSD1 and plastidic GhCSD2 were expressed at the highest during early fiber elongation, which may support the maintenance of low H$_2$O$_2$ levels (Kim *et al.*, 2008).

The maintenance of low levels of ROS during early elongation may occur during normal development, e.g., by dismutation of O$_2^•$ to O$_2$ and H$_2$O$_2$ by SOD and the oxidation of H$_2$O$_2$ to H$_2$O by APX. However, the role of ROS throughout elongation may be more complicated. First, ROS is increased throughout the fiber cell during late elongation as discussed above. Second, addition of H$_2$O$_2$ to cultured ovules increased fiber length (Li *et al.*, 2007) and ROS appears to be required for fiber elongation (Mei *et al.*, 2009). In addition to GhCSD1 and GhCSD2, other Cu/Zn-SOD genes, GhCSD3 and GhCSD3s, were expressed two-fold higher only in 16 DPA fiber and SOD proteins were localized to the primary and secondary wall (Kim *et al.*, 2008; Kim and Triplett, 2008). GhCSD3 may perform a particular role in the transition from elongation to secondary wall synthesis and will be discussed below. In contrast with the low levels of H$_2$O$_2$ maintained during early elongation, O$_2^•$ increased in fiber-bearing ovules during elongation from 0 to 10 DPA compared to fiberless fl ovules (Mei *et al.*, 2009). By the use of the ROS indicator 2, 7-DCH$_2$FDA, fluorescence was detected at 0 to 2 DPA while initiation and polar elongation occurred in wild-type, but no fluorescence was detected at -1 DPA in wild-type or at any DPA in ovules of the fl. When ovules in culture were treated with diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase activity, or a peroxidase inhibitor, salicylhydroxamic acid (SHAM), O$_2^•$ production and fiber length decreased in a concentration dependent manner in fiber-bearing ovules. The expression of another Class III plant peroxidase, GhPOX1, peaked at 10 to 15 DPA, which correlated with the peak in total peroxidase activity. GhPOX1 is homologous to AtPOX13, which was determined by mutant analysis to be required for lateral root initiation and elongation in arabidopsis. Therefore, GhPOX1 could have a similar role in cotton fiber. Although many peroxidases, such as APX, remove H$_2$O$_2$ from the cell, others produce ROS. GhPOX1 was proposed to produce ROS to promote fiber elongation (Mei *et al.*, 2009).

The exact mode of action for ROS is unclear at this time, but the study of ROS effects on the development of other plant systems may suggest avenues of research. For instance, ROS promote cell wall loosening and elongation in maize roots (Liszkay *et al.*, 2004) and the tip-growth of arabidopsis root hairs and tobacco pollen tubes (Monshausen *et al.*, 2007 and Potocký *et al.*, 2007, respectively). The dynamic nature of ROS during fiber elongation may support multiple modes of action for ROS or specific modes for different forms of ROS, i.e., O$_2^•$ and H$_2$O$_2$.

**Ca$^{2+}$ signaling during fiber elongation**

Ca$^{2+}$ signaling is a vital part of plant growth and development participating in responses to abiotic stress and phytohormones. Ca$^{2+}$ was a central player in the polar growth of root hairs and pollen tubes (Monshausen *et al.*, 2007; Cheung and Wu, 2008). Ca$^{2+}$ signaling also functions in cotton fiber polar growth and elongation. In ovule culture experiments, fiber elongation did not occur without Ca$^{2+}$ in the medium (Huang *et al.*, 2008). However, consistent with the fine control of cytosolic Ca$^{2+}$ levels required for Ca$^{2+}$ signaling, lower (0.1 mM) Ca$^{2+}$ promoted fiber initiation, early elongation, and the expression of expansin, compared to higher (1 mM) Ca$^{2+}$.
in ovule culture medium. The window of sensitivity to high Ca$^{2+}$ was the 0 to 3 DPA, but fiber elongation did not recover from early inhibition up to 10 DPA. The effects on fiber elongation of antagonists of Ca$^{2+}$ signaling in the presence of low or high calcium were consistent with Ca$^{2+}$ in the medium acting through signaling pathways, versus effects on other processes such as cell wall rigidity through pectin cross-linking (Taliercio and Haigler, 2011). Calmodulin is often a key component in Ca$^{2+}$ signaling pathways, and the calmodulin inhibitor, trifluoperazine, inhibited fiber elongation in vitro in a concentration dependent manner (Huang et al., 2008). Trifluoperazine inhibited fiber elongation more strongly in low calcium medium, which likely allowed more normal internal Ca$^{2+}$ concentration within fibers (Taliercio and Haigler, 2011).

Other components of Ca$^{2+}$ signaling have recently been characterized and shown to communicate with one another. Comparing gene expression in elongating fibers to leaves using a suppression subtraction cDNA library strategy identified fiber-specific/preferred genes involved in Ca$^{2+}$ signaling: calcineurin B-like (CBL) -interacting protein kinase (GhCIPK1), calmodulin (GhCaM), and glutamate decarboxylase (GhGAD). Each of these genes was expressed most strongly in 9 to 15 DPA wild-type fiber, but high expression ended at 9 DPA in fibers of the ligon lintless mutant that terminate elongation at <6 mm (Gao et al., 2007). GhCIPK1 functioned similarly to other CBL-interacting proteins during in vitro autophosphorylation kinase activity assays. Two CBLs, GhCBL2 and GhCBL3, that may interact with GhCIPK1 were also expressed in an elongation-specific manner, and both GhCBLs interacted with the C-terminus of GhCIPK1 in yeast two-hybrid and affinity pull-down assays (Gao et al., 2008). Calcium dependent protein kinases (CDPK) are another component of Ca$^{2+}$ signaling present in the elongating fiber. GhCDPK1 was expressed most strongly in 9 to 15 DPA wild-type fiber. Furthermore, GhCDPK1 protein fused to GFP localized in the plasma membrane of onion epidermal cells during transient transformation assays. GhCDPK1 performed autophosphorylation and phosphorylation of histone III-S only in the presence of Ca$^{2+}$, as expected for a CDPK (Huang et al., 2008). Calcium is also often involved in the cellular activity of annexins, which comprise a multifunctional protein family associated with Ca$^{2+}$-dependent membrane transport, GTPase activity, binding to filamentous actin (F-actin), and ROS reduction. GhFAnnx expression was ovule/fiber specific, and, in transient transformation assays, GhFAnnx-GFP fusions localized to the plasma membrane of onion epidermal cells in a Ca$^{2+}$-dependent manner and to the plasma membranes of cotton fibers after 14 days in culture (Wang et al., 2010c). In summary, fiber elongation was inhibited by interfering with Ca$^{2+}$ sensing, and the expression of proteins required for Ca$^{2+}$ signaling was correlated with the time of high-rate polar growth of fibers. Further work can be beneficially directed toward understanding connections within the Ca$^{2+}$ signaling pathway as well as connections with other signaling pathways.

**Emerging importance of fatty acids in fiber elongation**

Fatty acid metabolism is responsible for the biosynthesis of many cellular lipids especially membrane components, so rapid fatty acid synthesis in an elongating cotton fiber is expected. Indeed, genes required for fatty acid synthesis were highly expressed in elongating fiber (Shi et al., 2006). The total fatty acid content as determined by gas chromatography was also highest in elongating 7 to 21 DPA fibers compared to 28 DPA fibers. In the same study, cotton fiber EST libraries were
analyzed to implicate many genes that may be involved in de novo fatty acid biosynthesis in elongating fiber (Wanje et al., 2005). Fatty acids also provide building blocks of signaling molecules such as phosphoinositol and sphingolipids, and very-long-chain fatty acids (VLCFA) that often exist within sphingolipids have a particular role in fiber elongation. VLCFA accumulate preferentially in elongating fibers compared to ovules, and the cotton homologs of several genes supporting VLCFA biosynthesis (KCS12, KCS6, KCS13, and KCS2) were up-regulated in elongating fiber. The cotton genes were able to complement the yeast elo2Δ elo3Δ double mutant with defects in fatty acid elongase activity, confirming their function. When two types of VLCFA, C24:0 and C26:0, were added to cotton ovule culture, fiber elongation strongly increased compared to controls. In contrast, the inhibition of VLCFA biosynthesis by 2-chloro-N-[ethoxymethyl]-N-[2-ethyl-6-methyl-phenyl]-acetamide (ACE) led to a concentration dependent decrease in fiber length and ovule size (Qin et al., 2007a). GhCER6 also complemented yeast elo2Δ elo3Δ double deletion mutants for fatty acid elongase activity (Qin et al., 2007b). Other genes involved in VLCFA biosynthesis in cotton fiber include GhECR1 and GhECR2, which perform the reduction of trans-2-enoyl-CoA (ECR activity). These genes have expression peaks in elongating cotton fiber, although GhECR2 was more fiber-specific, and both could complement the yeast tsc13Δ mutant lacking ECR activity (Song et al., 2009). Three genes encoding 2-ketoacyl-CoA reductase (with KCR activity) were also expressed in cotton fiber. GhKCR1 and GhKCR2 have peak expression at 5 to 10 DPA and functionally complemented yeast ybr159wΔ mutants lacking KCR activity (Qin et al., 2005). GhKCR3 was expressed in 10 DPA fiber and also functionally complemented the yeast ybr159wΔ mutant. GhKCR3 had a higher affinity for long chain fatty acids (Pang et al., 2010b).

Experiments in cotton fiber suggest that VLCFA signal the expression of ET biosynthesis genes and ET accumulation with subsequent stimulation of fiber elongation (Qin et al., 2007a). Fibers on cultured ovules treated with combinations of VLCFA and ET and/or ET biosynthesis inhibitors showed that VLCFA-enhanced elongation occurred only if ET was provided exogenously or through biosynthesis. VLCFA addition to ovule cultures also led to higher ET and sphingolipid accumulation, and ET and sphingolipids levels decreased when VLCFA biosynthesis was inhibited. Analysis of the arabidopsis cut1 mutant in VLCFA biosynthesis using exogenous VLCFA or ectopic expression of GhKSC13 supported a role for VLCFA in elongation of roots and root hairs. VLCFA has been proposed to signal the expression of ET biosynthesis genes and ET accumulation with subsequent stimulation of fiber cell elongation (Qin and Zhu, 2010).

Significance of the cytoskeleton for fiber elongation

Cotton fiber elongation requires organized and efficient transport of new membranes and cell wall materials, processes that are aided by a dynamic cytoskeleton, including F-actin and microtubules. Genes associated with the cytoskeleton and intracellular transport had higher expression in microarray analysis in the chromosomal substitution line G. hirsutum cv. CS-B22sh compared to its progenitor (Wu et al., 2008). CS-B22sh contains a substitution of the chromosome 22 short arm from G. barbadense cv. 3-79 in the TM-1 genetic background, and it has improved lint percent, micronaire, and fiber length (Saha et al., 2006). This supports the impact of the cytoskeleton and intracellular transport on fiber quality.
During high-rate fiber elongation, microtubules are oriented transversely to the fiber axis and have a role in controlling the orientation of cellulose fibrils in cotton fiber (Seagull, 1993), as commonly occurs in plants (Paradez et al., 2006). At least nine b-tubulin genes are preferentially or differentially expressed in elongating fiber, and many of these, including *GhTUB1*, complemented a tubulin-deficient yeast mutant. Different combinations of these b-tubulin genes were stimulated in fibers of cultured ovules treated with ET, BR, GA, or VLCFA (He et al., 2008). The antisense suppression of the transcription factor *GhMYB109* led to decreased fiber length and suppressed expression of *GhACO1/GhACO2* that support ET biosynthesis, *GhTUB1*, and *GhACT1* (Pu et al., 2008). Therefore, key regulatory processes in cotton fiber ultimately exert their effects through the modulation of microtubules and downstream cellular effects related to the cytoskeleton.

Actin exists in thick microfilaments oriented axially and in a fine network in the cortical cytoplasm that sometimes parallels the microtubules during high-rate elongation (Seagull, 1993). The *in vitro* disruption of F-actin with cytochalasin D resulted in shorter fibers with a less rigid morphology (Wang et al., 2005; Wang et al., 2010d). Other recent work focused on the earliest phase of fiber elongation at 1 to 5 DPA when thick actin filaments (stained with rhodamine phalloidin) were arrayed parallel to the long axis of wild-type fiber (Li et al., 2005). Five of sixteen actin genes identified in cotton were expressed preferentially in fibers, *GhACT1, GhACT2, GhACT4, GhACT5*, and *GhACT11*. When *GhACT1* expression was reduced ~10-fold by RNAi in transgenic cotton, fiber length at 0 to 3 DPA was reduced 1.5- to 3-fold. The shorter 1 to 5 DPA fibers had fewer, more randomly arranged actin filaments compared to wild-type (Li et al., 2005).

The dynamic activity of the actin cytoskeleton is enabled by actin-modifying proteins. These include profilins (PFN), such as *GhPFN1* and *GhPFN2* that promote polymerization into F-actin, and actin depolymerization factors (ADF) such as *GhADF1*. Wang and coworkers worked extensively on fiber profilins, and there is evidence that *GhPFN1* may help to control fiber elongation (Wang et al., 2005; Wang et al., 2010d). *GhPFN1* was strongly expressed in 3 to 18 DPA fibers, and cultured tobacco cells constitutively over-expressing *GhPFN1* elongated more and had thicker, more abundant F-actin bundles compared to controls. *GhADF1*, expressed in fiber at 6 to 27 DPA, is likely to support actin depolymerization at multiple stages of fiber development. Down-regulation of *GhADF1* expression in transgenic cotton using RNAi resulted in less *GhADF1* protein and a heritable increase in fiber length (+5.6%) along with thicker, longer actin cables within fiber, presumably due to increased stability of actin in the presence of less actin depolymerizing protein (Wang et al., 2009).

**Primary wall structure during cotton fiber elongation**

Cotton fiber initiation and elongation depend on the rapid synthesis of a primary wall outside the plasma membrane. At a gross level, the cotton fiber primary wall is similar to those in other dicotyledonous plants (Doblin et al., 2010). It contains ~22% semi-crystalline (8-1, 4-glucan) cellulose fibrils, which are surrounded by a matrix composed mainly of other polysaccharides including xyloglucan and pectin (Meinert and Delmer, 1977; Singh et al., 2009a). The
high-strength cellulose fibrils are oriented transversely to the long fiber axis during elongation, which laterally constrains turgor pressure so that fiber elongation occurs. The elongating fiber wall must compromise strength and flexibility. For example, genes encoding wall-loosening expansin proteins (Sampedro and Cosgrove, 2005; Cosgrove, 2005) are expressed in rapidly-elongating fiber (Harmer et al., 2002) and are associated with QTLs related to fiber length (An et al., 2007).

The presence and modification of the matrix components also contribute to cell wall properties and growth potential (Cosgrove, 2005) and may regulate fiber elongation, as will be discussed further below. Pectins are a complex family of galacturonic acid-rich polysaccharides including: (a) homogalacturonan, with/or without side-groups or side-chains; and (b) rhamnogalacturonan I with a repeating disaccharide (galacturonic acid-rhamnose) in its backbone and a wide variety of side-groups or side-chains (Mohnen, 2008). Xyloglucan has a β-1, 4-glucan backbone with a variable pattern of side-chains composed of α-1, 6-xylose and sometimes galactose and fucose (O’Neill and York, 2003). Research is continuing on how cellulose and the matrix components work together through largely non-covalent interactions to generate strength, flexibility, and developmental plasticity within the composite primary wall (e.g., Abasolo et al., 2009; Boyer, 2009).

Specialization of cell walls is often a key feature of cellular differentiation (Cosgrove, 2005), and changes in cell wall structure and chemistry are a signature of the progression of fiber development. Experimental or breeding lines with differences in fiber quality frequently show differential gene expression related to cell walls and/or related processes such as cytoskeletal organization (e.g., Wu et al., 2008; Hinchliffe et al., 2010). A montage of micrographs in Seagull (1993) shows the changes in the orientation of cellulose fibrils in successive cell wall layers as fiber development proceeds, and equally dramatic changes occur in cell wall matrix components. Recently, immunolabeling of cotton fiber from wild-type and fiberless lines with antibodies raised against isolated rhamnogalacturonan I showed that cotton initiation and early elongation at 0 to 2 DPA were characterized by: (a) loss of an epitope characteristic of (1–6)-β-D-galactan carrying arabinose (possibly contained in situ within arabinogalactan protein); and (b) appearance of an epitope characteristic of (1–4)-β-D-galactan, one of the possible side chains of rhamnogalacturonan I (Bowling et al., 2011). This study also confirmed the existence of an outer pectin-rich sheath surrounding an inner primary wall that was enriched in cellulose and xyloglucan, as described previously (Vaughn and Turley, 1999). This outer pectin sheath is not known in other plant cells, demonstrating that aspects of cotton fiber cell wall structure are likely to help to control the differentiation program and unique features of cotton fiber.

**Pectin synthesis and modification as regulators of cotton fiber elongation**

The importance of pectin in modulating cotton fiber elongation has been shown in two recent studies. Pang and coworkers (Pang et al., 2010a) compared 10 DPA whole ovules of wild-type and the fuzzless-lintless cotton mutant by proteomics, which implicated proteins associated with nucleotide sugar metabolism and pectin biosynthesis as supporting fiber elongation. In
ovule culture experiments, transcripts of the pectin biosynthesis genes responded positively to the addition of ET or a VLCFA (lignoceric acid, C24:0, C23H47COOH), which are positive regulators of fiber elongation (Qin et al., 2007a) as described previously. Fiber elongation in culture also responded positively to the addition of UDP-activated rhamnose, galacturonic acid, or glucuronic acid, and UDP-rhamnose or UDP-galacturonic acid allowed fiber elongation in the presence of an ET perception inhibitor. Together with experiments on arabidopsis mutants with defects related to pectin biosynthesis, C24:0 biosynthesis, or ET signaling, these results supported the model that pectin biosynthesis was a target for signaling pathways that promote fiber elongation (Pang et al., 2010a). A positive correlation between cell expansion and pectin supply occurs frequently in plants (Boyer, 2009).

In addition, the molecular structure of pectin is critically important for cotton fiber elongation. Wang and coworkers (Wang et al., 2010a) studied the effect of increased levels of de-esterified homogalacturonan in elongating cotton fiber. They showed that a gene encoding an authentic pectate lyase, GhPEL, was preferentially expressed in fibers with peak expression at 10 DPA. The recombinant GhPEL protein was able to degrade polygalacturonic acid (de-esterified homogalacturonan) in vitro. Peak pectate lyase activity occurred in wild-type fiber at 10 DPA, and the amount of de-esterified pectin (in a crude extract that could contain other cell wall polymers) declined continuously from 5 to 20 DPA in wild-type fiber. The 10 to 15 DPA fiber of transgenic cotton with reduced GhPEL expression had less pectate lyase activity and increased content of (putative) de-esterified pectin. Mature transgenic fiber was proposed to be about 3 – 16% shorter across several homozygous lines, and reduced fiber elongation beginning at 10 DPA occurred in one line compared to wild-type. Potentially shorter cotton fibers when the amount of de-esterified homogalacturonan increased could be explained by more pectin gel formation leading to cell wall rigidification, a phenomenon that occurs when calcium acts as a cross-linker for de-esterified homogalacturonan (Cosgrove, 2005; Boyer, 2009).

**Xyloglucan modification as a regulator of cotton fiber elongation**

Many researchers have explored the potential of xyloglucan to help regulate plant cell growth. Surprisingly, xyloglucan-depleted arabidopsis plants grew almost normally in the laboratory, although cell wall mechanical properties were changed and root hairs were short with bulging bases (Cavalier et al., 2008). For cotton researchers, the main targets of exploration have been a family of xyloglucan endo transglycosylase/hydrolase (XTH) genes encoding proteins that have one or both abilities to degrade xyloglucan irreversibly (xyloglucan endo-transglycosylase, XET, activity) or to cleave and transfer chain ends between molecules (xyloglucan endohydrolase, XEH, activity). In species characterized so far, this is a large gene family and each encoded enzyme must be analyzed individually in biochemical and cell biological experiments to determine its actual function (Eklöf and Brumer, 2010). Modulations in one or both of the XET/XEH enzyme activities could possibly increase the plasticity of the primary wall, thereby promoting fiber elongation (Cosgrove, 2005). Several XET/XEH genes had expression peaks during cotton fiber elongation (Michailidis et al., 2009; Lee et al., 2010) and/or at the transition stage of fiber development, possibly with a relationship to CFML degradation (Singh et al.,
2009a; see below for further discussion of the CFML). Supporting the positive impact of XTH genes on fiber elongation, longer domesticated cotton fiber showed higher expression of several of these genes as compared to wild *G. hirsutum* (Rapp *et al.*, 2010). Proving the importance of XET activity for fiber elongation, analysis of transgenic cotton plants over-expressing *GhXTH1* under the control of the constitutive CaMV 35S promoter showed a positive correlation between inheritance of the transgene and increased fiber length. The transgenic plants had about two-fold increased XET activity and 15-20% longer fiber compared to wild-type cotton or null segregants under greenhouse or field conditions, leading to the conclusion that the ability to transfer xyloglucan chain ends between molecules was a limiting factor for fiber elongation in *G. hirsutum* cv. Coker 312 (Lee *et al.*, 2010).

**Existence and effects of a cotton fiber middle lamella**

In addition to the primary wall layers that are closest to the protoplast, cotton fiber has an outer primary wall layer called the cotton fiber middle lamella (CFML). The CFML joins adjacent elongating fibers together through forming a unified cell wall between them (Singh *et al.*, 2009a). Fibers are not merely in superficial surface contact; instead they are joined with their neighbors into tissue-like bundles just as other cells form tissues within the plant body. Given that cotton fibers initiate from the ovule as individuals and are subsequently found within the open boll as individuals, the discovery of fused cotton fibers during elongation was surprising. The CFML is enriched in fucosylated xyloglucan and homogalacturonan pectin with a relatively low degree of esterification, and it may contain other unidentified components that help to confer its adhesive properties. The CFML may derive from the pectin-rich outer layer of the primary wall described for 1 to 2 DPA fibers, although xyloglucan was not detected in this outer pectin-rich fiber “sheath” just after initiation (Vaughn and Turley, 1999). The fiber bundles adopt curving paths as elongation proceeds finally creating a tight packet of fibers around each seed. This packet of fiber grows outward until it contacts the boll wall by 3 to 5 DPA. The CFML-mediated formation and packing of fiber bundles explains “spiral structures” described for cotton fiber (Krakhmalev and Paiziev, 2006). This high level of organization of fiber within the boll probably facilitates the elongation of thousands of fibers within a confined space. The CFML is degraded by cell-wall active enzymes during the transition to secondary wall deposition while the inner layer of the primary wall is left relatively intact. This causes the fibers to be released as individuals at the onset of secondary wall deposition (Singh *et al.*, 2009a), although the packing pattern of fiber within the locule does not change until the boll opens.

**Transitional Primary Wall Remodeling and Winding Layer Synthesis**

The targeted degradation of the CFML, and possibly other primary wall remodeling, at the transition stage provides at least partial explanation for changes in the concentrations of primary wall sugars (Meinert and Delmer, 1977; Singh *et al.*, 2009a). The transition stage of fiber development is often thought of as a period when primary and secondary wall deposition overlap. It
is more accurate to consider it as a separate stage of fiber development that controls cotton fiber quality in important ways. During the transition stage many distinct cellular events occur. The rate of respiration in the fiber/ovule system goes through a trough before rising again during secondary wall synthesis; intra-fiber sugar pools decline; fiber cellulose synthesis occurs at an intermediate rate; the percentage of cellulose in the fiber cell wall increases to ~35%; and the CFML degrades. At the same time, the amount of callose, or β-1,3-glucan, in the fiber reaches its peak (Meinert and Delmer, 1977; Maltby et al., 1979; Martin and Haigler, 2004; Guo et al., 2007; Singh et al., 2009a; Abidi et al., 2010b).

In addition, the cytoskeletal microtubules just below the fiber plasma membrane reorient from their transverse state during fiber elongation to adopt a shallow helix relative to the fiber axis. The dynamic behavior of F-actin is a potential regulator of the timing of the transition stage. In control plants, the profilin gene, GhPFN2, was highly expressed at 15 to 24 DPA, and GhPFN2 promoted actin bundling in the presence of an arabidopsis formin, AtFH1. Formin proteins interact with PFN and actin to aid F-actin formation. Cotton over-expressing GhPFN2 had shorter fibers and initiated secondary wall deposition 2 days earlier than control plants (Wang et al., 2010d). Another gene encoding an actin depolymerization factor, GhADF1, was expressed at 6 to 27 DPA and is likely to support actin depolymerization at multiple stages of fiber development. Transgenic cotton with down-regulated GhADF1 expression and less GhADF1 protein showed a heritable increase in fiber length (+5.6%) along with thicker, longer actin cables within fiber. Other phenotypes included higher cellulose content at 24 DPA (~95% compared to ~91% in the control) and a thicker secondary wall (Wang et al., 2009). Together these results show a positive role for F-actin in initiation of secondary wall thickening.

The “winding” cell wall layer, reflecting the first phase of wall thickening, is synthesized with a lattice-like organization of fibrillar elements. The cellulose microfibrils in the winding layer parallel the reoriented microtubules with a shallow angle relative to the fiber long axis (Fig. 1E; reviewed in Seagull, 1993). The change in microfibril angle as compared to the primary cell wall is analogous to creating plywood, and probably explains the fact that a substantial degree of final fiber strength is conferred during the transition phase (Hsieh et al., 1995; Hinchliffe et al., 2011) even though the increase in cell wall mass is not extensive. During the time that the cell wall begins to thicken, changes occur in the degree of esterification of primary wall pectin that may serve to limit elongation potential. In addition, a cuticle that stains darkly in sections viewed in TEM begins to integrate with the primary wall (Singh et al., 2009a). (Singh and co-workers discuss the evidence for synthesis of the cotton fiber cuticle at the transition stage and not during fiber elongation). The primary wall and cuticle are pushed to the perimeter of the fiber as cell wall thickening occurs at the plasma membrane.

As expected, significant changes in gene expression and potential regulatory processes occur at the transition stage. In G. hirsutum, there is a decline in the expression of primary wall-related genes and the increased expression of secondary wall-related genes (Hinchliffe et al., 2010 and other references therein). In addition, some genes have distinct peaks of expression at the transition stage. These may encode proteins that affect cellular processes directly (e.g., Singh et al., 2009a) or underpin the signaling events that control the timing of the transition phase. For example, GhRAC13, a small GTPase, may contribute to an oxidative burst that stimulates the
onset of secondary wall deposition (Potikha et al., 1999; Yang et al. 2008). This signaling event may be further controlled through transition stage up-regulation of SOD to act as a scavenger of ROS in the cell wall. The expression patterns of four Cu/Zn-SOD genes have been analyzed in fiber. Two of these, GhCSD3 and GhCSD3a, were expressed two-fold higher only in 16 DPA fiber, SOD specific activity peaked at 24 DPA, and SOD was localized in the primary and secondary wall. The authors proposed that altered levels of ROS could signal changes in gene expression related to cell wall synthesis and/or affect cell wall properties directly through effects on cell wall rigidity (Kim et al., 2008; Kim and Triplett, 2008).

The timing of the transition phase can be changed by genotype or environment and impacts cotton fiber quality. Microscopic and gene expression analysis in cultured fiber showed that natural auxin (IAA) accelerates entry into the transition phase and the onset of high-rate cellulose synthesis as compared to synthetic auxin (NAA) (Singh et al., 2009b). Thermogravimetric analysis (fiber weight vs. exposure to high temperature) showed that the transition phase started either at 17 to 18 DPA or 21 to 24 DPA in two greenhouse-grown G. hirsutum cultivars (Abidi et al., 2010a). In a highly regulated greenhouse with a relatively cool 26/22°C day/night cycle, secondary wall-related genes were up-regulated by 19 DPA in fiber of G. hirsutum cv. Deltapine 90 (Singh et al., 2009b), which is later than often observed in the field. A study of two G. hirsutum near-isogenic lines, one with consistently higher (~15%) fiber bundle strength attributable to one or two genes, clarified a major reason why the timing of the transition stage varies (Hinchliffe et al., 2010; Hinchliffe et al., 2011). Over several field seasons, fiber of these lines showed > two-fold higher expression of genes related to secondary wall synthesis as early as 12 DPA or later than 18 DPA (in a season with cool night temperature). Hinchliffe and coworkers determined that the timing was governed by temperature: the transition started when 160 degree day heat units were accumulated after anthesis (calculated from daily high and low temperatures using a threshold of 15.5°C). This relationship held true for plants grown in the greenhouse by Singh and coworkers (Singh et al., 2009a). Furthermore, entering the transition stage earlier resulted in higher fiber bundle strength, for reasons that remain to be discovered. These results show the importance of identifying potential master control genes for the onset of the transition stage in cotton fiber (Hinchliffe et al., 2010; Hinchliffe et al., 2011).

Secondary Wall Synthesis

Cotton fiber secondary wall thickening occurs through the deposition of nearly pure cellulose. So far no other component has been characterized that might exist between the cellulose fibrils in the unique cotton fiber secondary wall. The percentage of crystalline cellulose was 90% in wild-type fiber with a maturity ratio of 0.89 as measured by image analysis of fiber cross-sections. In transgenic fiber with similar fiber perimeter, the percentage of crystalline cellulose increased to nearly 92% along with an increase in the maturity ratio to 0.95-0.99 (Haigler et al., 2007). After chemical-dehydration and sulfuric-acid extraction, ~85% or 90% percent cellulose was found for the fiber of two other G. hirsutum cultivars (Abidi et al., 2010b). All of these values may under-estimate the crystalline cellulose content of the isolated secondary wall because: (a) the whole fiber including the cuticulated primary wall was included in the fiber weight; and
(b) the acids used as extractants may have dissolved some less ordered cellulose. Therefore, the cotton fiber secondary wall likely contains >95% cellulose, making it the purest cellulose synthesized by plants. Secondary wall thickening is characterized by an increased rate of cellulose synthesis, greater length of individual cellulose chains, and orientation of closely packed cellulose fibrils in an increasingly steep helix relative to the fiber axis (Fig. 1F). The cellulose microfibrils also reverse their direction of helical travel at intervals, creating “reversals” within the secondary wall structure (reviewed in Seagull, 1993).

The cellular, biochemical, and genetic regulation of cellulose synthesis are key aspects of cotton fiber differentiation, but the details are beyond the scope of this review. Broadly, the cellulose synthase genes and other co-functional genes that support cotton fiber secondary wall cellulose synthesis are orthologs of those required for secondary wall deposition in the xylem of vascular plants, whereas cotton fiber elongation and the wall thickening of arabidopsis leaf trichomes are supported by genes related to primary wall synthesis (Betancur et al., 2010). This and other evidence supports the idea that a process for xylem secondary wall cellulose synthesis at the base of the land plant lineage was widely adapted in other cells as plants evolved (Haigler et al., 2009; Zhong et al., 2010; Hinchliffe et al., 2010). The conservation of the secondary wall cellulose synthesis genetic program is also indicated by comparing gene transcription in wild vs. domesticated G. hirsutum, which showed less difference between genotypes during secondary wall synthesis as compared to elongation (Rapp et al., 2010). However, cotton fiber employs the basal xylem secondary wall thickening program in a unique way given that hemicellulose and lignin synthesis do not occur while nearly pure cellulose is synthesized in cotton fiber. Nonetheless, rather than leaf trichomes, the best models in arabidopsis for cell wall thickening in cotton fiber are xylem conducting cells or other cellulose-rich schlerenchyma cells (Betancur et al., 2010).

How the cotton fiber supports and regulates the strong irreversible carbon sink represented by secondary wall cellulose synthesis has been reviewed previously (Haigler et al., 2001; Delmer and Haigler, 2002; Haigler, 2007). A particular focus of research has been the possibility that the UDP-glucose substrate for cellulose synthesis is generated as sucrose is cleaved by sucrose synthase. Cryogenic TEM sample preparation methods that disallowed protein movement showed that Sus was located near the plasma membrane and in cotton fiber secondary walls (Salnikov et al., 2003). The cell wall location was unexpected but subsequently confirmed by others (Ruan, 2007; Brill et al., 2011). Brill and coworkers identified and characterized a novel Sus isoform, SusC, with divergence outside the catalytic region from other Sus proteins. SusC gene expression was up-regulated during cotton fiber secondary wall cellulose synthesis, and SusC together with other Sus isoforms were found in the apoplast as well as intracellularly. The function of apoplast Sus in cotton fiber remains to be determined. It is potentially involved in synthesis of apoplastic callose that was co-distributed with Sus (Salnikov et al., 2003) and/or cellulose synthesis (Brill et al., 2011). Additional evidence for the importance of sucrose in cotton fiber secondary wall deposition is provided by transgenic plants constitutively over-expressing spinach sucrose phosphate synthase, a key regulator of the rate of sucrose synthesis (Haigler et al., 2007). Several independent transgenic cotton lines had higher leaf sugar content. One line with the highest level of spinach sucrose phosphate synthase protein in leaf and fiber
produced fiber with thicker secondary walls (compared to wild-type and a null segregant) when grown in the growth chamber with cool nights. Cool temperatures are well known to hinder the rate of cellulose synthesis and fiber wall thickening in wild-type cotton (Haigler et al., 2007 and references therein). In related results, several lines of transgenic cotton over-expressing mustard annexin had higher fiber cellulose content compared to wild-type under salt stress conditions. The mature fiber phenotype was correlated with salt-induced up-regulated expression in leaves and fiber of genes encoding sucrose phosphate synthase, sucrose synthase, and cellulose synthase (Divya et al., 2010).

**Fiber Maturation**

The signal to end secondary wall cellulose synthesis is unknown, but afterwards the terminal, ill-defined maturation stage of fiber development begins. Difficulty in extracting protein and nucleic acids from late stage fiber hinder mechanistic studies (Kim and Triplett, 2001). Analogously to xylem tracheary elements, programmed cell death mechanisms may also operate during this time, at least partly controlled by ROS (Potikha et al., 1999; Kim and Triplett, 2001). Cellular hallmarks of programmed cell death mechanisms include nuclear blebbing, DNA and cytoplasmic degradation, and caspase-like activity (Love et al., 2008); however, attempts to document DNA degradation were inconclusive for cotton fiber (Roche, 2009). Soon after boll opening, the cotton fiber dries and collapses into the kidney bean cross-sectional shape that aids spinning into yarn, assuming that the fiber has an optimal maturity ratio. The fiber of many seeds fluffs into the mass typical of the open cotton boll (Fig. 1I, J).

**SUMMARY**

The cotton fiber is economically important and a significant tool for understanding cellular, biochemical, and molecular processes at the single-cell level. Advances in understanding fiber development are being made more rapidly now through research centered on functional genomics and cell biology. Virus induced gene silencing in cotton fiber should help future research of this type to progress faster (Tuttle et al., 2008; Idris et al., 2010; Tuttle and coworkers, in preparation for publication). Results from proteomics and metabolomics are starting to appear and will undoubtedly increase in the future. The description of transcriptional regulators such as miRNA and transcription factors could soon lead to an integration of their effects into a larger model of fiber gene expression regulation. Likewise, the genetic and physiological data on the roles of individual growth regulators such as ET and BR should be integrated to highlight their mechanisms. Further study of nearly 100% cellulose synthesis in cotton fiber is expected to be applicable to altering cell wall properties in cotton and other plants, including biomass crops. Each proof of a control point of fiber morphogenesis increases the potential to devise sophisticated strategies to improve fiber quality beyond the remarkable characteristics already conferred through evolution, domestication, and traditional breeding. Next generation cotton germplasm with higher fiber yield and quality, as well as potentially novel fiber characteristics, will be an important part of providing renewable, sustainable, resources to a growing human population.
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