Cotton Fiber Growth in Planta and in Vitro. Models for Plant Cell Elongation and Cell Wall Biogenesis

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There are only a few cells in the plant kingdom that are as exaggerated in their size or composition as cotton fibers. It is precisely their highly elongated structure and exceptional chemical make-up that establishes cotton fiber as an ideal model for studies of plant cell elongation and cell wall biogenesis. Cotton fibers are unicellular, therefore cell elongation can be evaluated independently from cell division. Although commonly called fibers, the more botanically appropriate term is trichome, since these cells are not part of the vascular tissue and arise, instead, from the ovule epidermis.

ADVANTAGES OF COTTON FIBER DEVELOPMENT IN PLANTA

The economically important seed trichomes of *Gossypium hirsutum*, the cotton variety most commonly grown in the United States, typically range in length from 2.2 to 3.0 cm. Fibers from another commercially important species, *Gossypium barbadense*, may reach lengths of over 6 cm, or one-third the height of an Arabidopsis plant. Fiber cells from *G. hirsutum* range in diameter from 11 to 22 μm and are, therefore, 1,000 to 3,000 times longer than they are wide. Cotton fibers are linear cells and are never branched like many leaf trichomes. In addition to being among the longest plant cells ever characterized, a single cell wall biopolymer, cellulose, accounts for more than 95% of the dry weight of mature cotton fiber. Unlike many plant secondary cell walls, the cotton fiber wall contains no lignin. Typical of many plant cells, cotton fibers have a large central vacuole that becomes prominent quite early in development.

Cotton fiber development consists of four overlapping developmental stages: fiber initiation, cell elongation, secondary wall deposition, and maturation. Initiation of fiber development is conveniently timed beginning on or near the day of anthesis. Approximately 25% of the ovular epidermal cells differentiate into the commercially important lint fibers. Fiber initiation and cell elongation are fairly synchronous on each ovule and among the approximately 25 to 30 ovules per ovary (boll). Several days later, another class of fiber cells, fuzz fibers or linters, starts growing, but for unknown reasons these cells rarely become longer than 15 mm. With each ovule supporting the growth of approximately 13,000 to 21,000 lint fiber cells, a single ovary contains about one-half million synchronously elongating cells representing a single plant cell type.

Cell expansion continues from the day of anthesis to approximately 21 to 26 DPA. Fiber growth occurs by intercalation of materials throughout the fiber length and by tip growth (Seagull, 1990a). Solute movement into the fiber cell during cell expansion was shown recently to result from developmentally reversible gating of plasmodesmata (Ruan et al., 2001). Amplification of nDNA has been measured in fiber cells up to 5 DPA (van't Hof, 1999) and may occur over a longer period. Endoreduplication is frequent in other plant and animal cells prior to cell enlargement and is known to occur during Arabidopsis leaf trichome differentiation (Szymanski and Marks, 1998). The identity of genes that are amplified is unknown in both systems, however in cotton fiber the nucleolus expands in volume during this time period suggesting that ribosomal sequences may be among the amplified genes (DeLanghe et al., 1978).

After slightly more than 2 weeks of lengthening, fiber cells synchronously enter the third stage of development, secondary wall deposition. During this time, the β-1,4-glucan chains that form the cellulose microfibrils of the secondary wall are synthesized. Successive layers of cellulose are deposited until the wall is 3 to 4 μm thick. Cellulose microfibrils are arranged helically around the growing fiber with periodic changes in the deposition angle. The reversal regions, where cellulose microfibril orientation changes, cause the mature fiber to twist. Without fiber twist the individual fibers could not be spun into yarns. The maturation phase of fiber development has not been investigated in detail largely due to low protein and nucleic acid recovery from cells encased in a thick secondary cell wall. There has been much speculation that fiber differentiation may be similar to xylem tracheary element differentiation and involve programmed cell death. To date, however, there have been no published reports showing

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nuclear degradation, vacuole rupture, or any of the other molecular and biochemical markers for programmed cell death in maturing cotton fiber.

At approximately 45 to 60 DPA, the seed capsule dehisces and the thin fiber cells quickly dehydrate. As the cytoplasm dries, it adheres to the innermost layer of the fiber cell wall leaving a lumen where the central vacuole was once located. After harvest, removal from the seed, and other mechanical processing steps, the exocellular matrix of this once living cell becomes a versatile natural fiber for textile and other uses.

Clearly one advantage to the use of cotton fiber as an experimental model is that it is an important commodity worldwide. Over 90 million bales of cotton (2×10^{10} kg) are expected to be harvested from the major cotton-producing nations in 2001. As a result of competition from man-made textile fibers and new innovations in textile processing machinery, there is great interest in improving the quality and yield of cotton. Specialized instruments to grade cotton for commerce are available that measure the length, strength (tenacity), fineness, and maturity of fiber bundles. Also, several instruments designed to test single-fiber properties are available in limited numbers for research purposes. Use of these instruments with developing fiber cells has proven useful toward understanding relationships among fiber development, structure, chemistry, and physical properties (Hsieh et al., 1997, 2000).

Although field production of this crop is limited to areas where the growing season is at least 135 d with night temperatures greater than 18°C to 20°C, greenhouse production for research purposes is possible in cooler climates (Beasley, 1974). Nevertheless, in the same time that Arabidopsis can develop from seed-to-seed, cotton plants are just beginning to flower. Cotton is a perennial plant with indeterminate growth, so flowers initiate continually during the growing season beginning on the lower branches. Multiple floral buds, called squares, may be supported on each sympodial branch.

Due to the high cellulose content in cotton fiber cell walls, it is no surprise that the first subunits of plant cellulose synthase to be cloned (CesA1/CesA2) came from sequence comparisons of cotton fiber genes expressed during secondary cell wall formation with bacterial cellulose synthases (Pear et al., 1996). New insights into the biochemistry of cellulose biosynthesis and identity of other subunits of this enzyme complex are likely to come from Arabidopsis cellulose biosynthetic mutants such as *radialswelling1* (Arioli et al., 1998), *irregular xylem 1* (Taylor et al., 2000), and *korrigan* (Lane et al., 2001) and from other bioinformatic approaches with Arabidopsis. There are, however, some features of β-glucan biosynthesis for which biochemical studies on cotton fiber (Peng et al., 2001) or analysis of cotton fiber gene expression may be the most useful approach. For example, cellulose molecules in the primary cell wall have a lower M_r distribution than cellulose molecules deposited in the secondary wall (Marx-Figini, 1982; Timpa and Triplett, 1993). Forthcoming models of cellulose synthase will need to account for such differences and cotton fiber will be a good model for such studies. Also, a small amount of callose, a β-1,3 glucan, is deposited in fiber cell walls and remains detectable near the plasma membrane throughout the secondary wall-thickening stage (Waterkeyn, 1981). As a result, cotton fiber is ideally suited for comparing the regulation and structure of plant cellulose and callose synthases (Cui et al., 2001).

One might expect that the high cellulose content of cotton fiber cell walls resulted from a plant cell that was exclusively dedicated toward this end. In addition to genes involved in cellulose production, there is a remarkable diversity in the type and number of genes expressed in fiber throughout all developmental stages (Graves and Stewart, 1988; Ferguson et al., 1996). All levels of genetic regulation from transcriptional to post-translational control seem to be operating during fiber development. Based on the large number of non-normalized ESTs deposited in GenBank for early stages of fiber development (M. Blewitt, E.C. Matz, and B. Burr, unpublished data; R.A. Wing, D. Frisch, Y. Yu, D. Main, T. Rambo, J. Simmons, D. Henry, T.C. Wood, A. Leslie, and T.A. Wilkins, unpublished data; Y.Q. Zhu, K.X. Xu, J.W. Wang, and X.Y. Chen, unpublished data), the number of genes expressed in cotton fiber is probably no different than the number expressed in most plant cell types. Numerous full-length genes from developing cotton fiber have been cloned and expression patterns characterized (John and Crow, 1992; Delmer et al., 1995; John and Keller, 1995; John, 1996; Pear et al., 1996; Reinhart et al., 1996; Ma et al., 1997; Shimizu et al., 1997; Song and Allen, 1997; Kawai et al., 1998; Smart et al., 1998; Loguercio et al., 1999; Whittaker and Triplett, 1999; Orford and Timmis, 2000; Cui et al., 2001). From these studies it appears that there are some fiber genes expressed only during initiation and cell elongation phases of development, other genes that are expressed only during secondary wall thickening, and a third class of fiber genes that are constitutively expressed throughout fiber development. In the near future, high-throughput methods will rapidly accelerate the pace with which fiber gene expression can be monitored.

Despite the advantages of using cotton fiber as a model for primary cell wall structure, there have been many fewer studies on fiber non-cellulosic polymers compared with studies on cellulose (Meinert and Delmer, 1977; Huwyler et al., 1979; Hayashi and Delmer, 1988). As a result, there are no currently accepted models for the structure of fiber primary cell walls. This oversight is striking since the fiber

primary wall and cuticle must be removed for cotton textiles to be processed. The ease with which substantial quantities of developmentally staged primary cell walls can be isolated from plants or ovule cultures makes cotton fiber an ideal model for dicot primary cell walls. Additional details about the composition and structure of cotton fiber primary cell walls and changes in composition and structure that occur during fiber development could contribute significantly to working models of plant cell expansion.

A limited number of near-isogenic fiber-development mutants are known (Kohel et al., 1974). These mutants were not induced but, rather, arose spontaneously in cotton breeding programs over many years. Fiber cell length is reduced to 4 mm in the Ligon-lintless (*Li1, Li2*) mutants. In naked seed (N_1) there are no short fuzz fibers and a substantially reduced number of lint fibers. The pilose mutant (H_2) results in shorter, thicker fibers and a very dense distribution of leaf trichomes. Interestingly, there appears to be genetic linkage between leaf trichome density and quantitative trait loci affecting fiber fineness (Kloth, 1995). The immature fiber mutant (*im*) does not produce a fully mature secondary cell wall. In addition to these recognized developmental mutants, the genus *Gossypium* contains over 30 recognized species with widely diverse fiber characteristics.

ADVANTAGES OF COTTON FIBER DEVELOPMENT IN VITRO

One of the most significant benefits for using cotton fiber as a model system for plant development is that a culture method for cotton ovules was perfected three decades ago (Beasley, 1971; Fig. 1A). Day of anthesis cotton ovules will produce fiber when floated on the surface of a defined medium (Beasley and Ting, 1973). If fertilized ovules from 2 DPA or older are used, cultures will also support the growth of developing embryos (Beasley and Ting, 1974). Fiber development is dependent on the addition of phytohormones. Both auxin (indole acetic acid or naphthalene acetic acid) and gibberellic acid must be added to cultures initiated with day of anthesis ovules. Cytokinins, abscisic acid, and ethylene are inhibitory to fiber development.

On the day of anthesis each cotton ovule is approximately 1.5 mm long and therefore 60 times larger than a mature Arabidopsis seed. These large, easily manipulated ovules can be harvested from dozens of ovaries for well-replicated experiments. For RNA isolation, protein characterization, fiber length, or cellulose content measurements, sufficient quantities of fiber are produced in three or four cultures. In our laboratory, cultures are grown in 100- × 25-mm petri dishes in the dark at 32°C in a 5% CO_2 incubator. The overall quality of the cultures is enhanced by exposure to CO_2 (Xie and Stewart, 1989) perhaps by more closely reflecting physiological conditions inside the ovary (Jacks et al., 1993).

Ovule cultures have obvious advantages over whole plants when experimental protocols call for inhibitors, radiolabeled precursors, or controlled environmental conditions to be tested. Although fibers produced in culture thus far do not reach lengths achieved by intact plants, frequently fibers grow to 1.7 cm with cellulose compositions of 70% to 80%. Early comparison suggested substantial similarity between the fibers produced in planta and in vitro (Meinert and Delmer, 1977). More recent studies sug-

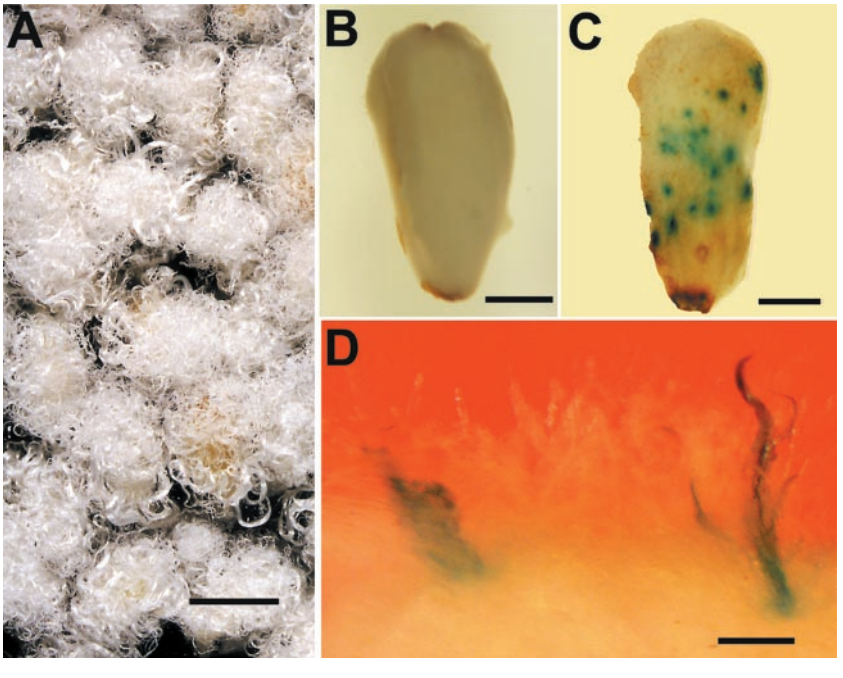

Figure 1. A, Cotton ovule culture 21 d after culture initiation; B, non-transformed, day of anthesis cotton ovule stained for GUS expression; C, day of anthesis cotton ovule transformed by particle bombardment 12 h after transformation with 35S-CaMV:GUS and stained for GUS expression; and D, transient expression of GUS in fibers produced in ovule culture. Scale bar in A = 1 cm, in B and C = 500 μm, in D = 50 μm.

gest that fibers developing in culture may differ from fibers produced by intact plants in the degree of branching of carbohydrate polymers (Triplett and Timpa, 1995) and in their protein profiles (Turley, 1998).

The relationship of the plant cytoskeleton with cell wall polymer organization is an active area of investigation by many plant scientists. Cotton fibers produced in ovule culture are uniquely suited to study the plant cell wall-cytoskeleton continuum. The organization of cortical microtubules and some of the actin microfilaments parallels the orientation of cellulose microfibrils in cultured fiber cells (Seagull, 1990b). In addition, re-organization of the fiber cytoskeleton occurs concomitantly with the initiation of secondary wall synthesis (Seagull, 1986). Cytoplasts or anucleate protoplasts from cotton fiber cells are easily obtained in large numbers starting with fibers grown in vitro (Gould et al., 1986). Cytoplasts proved useful in the purification of cotton-fiber cytoskeletal preparations (Andersland et al., 1998; Andersland and Triplett, 2000) and may be an excellent starting material for purification of other subcellular components involved in cell wall expansion.

Fibers produced in planta are unicellular and always cease dividing before the fiber cell has started expanding. When 2 DPA ovules are cultured in hormone-free media, in vitro fibers continue to divide for several days (van't Hof and Saha, 1997). The production of multicellular fibers under these altered culture conditions suggests that phytohormones may be involved in the establishment of a barrier to additional cycles of cell division after fiber cell elongation has started. The nature of this inhibition is unknown but is worthy of investigation since cessation of the cell cycle typically is linked to the cell's commitment to differentiate. *SIAMESE*, an Arabidopsis mutant that produces clusters of trichomes is known to continue cell division during trichome formation (Walker et al., 2000).

In addition to fiber development, cotton ovule cultures are quite useful for studying early stages of dicot embryogenesis. Fertilization is assured if ovules are harvested from plants at 2 DPA and placed into ovule culture. By 14 to16 DPA, embryos will develop to the early cotyledonary stage in 30% to 50% of the cultures. Rescue of interspecific hybrids using ovule culture has been successful when the nitrogen content of the medium was changed from the standard Beasley-Ting protocol (Stewart and Hsu, 1977, 1978).

In the last 30 years, cotton ovule cultures have been used by numerous investigators for a number of other applications (Triplett, 2000). Whether for analysis of suboptimal temperatures on cellulose biosynthesis (Haigler et al., 1991; Xie et al., 1993), looking at plant-fungal interactions (Mellon, 1986), or examining the structure and biochemistry of naturally pigmented cotton fibers (Ryser et al., 1983), cotton ovule cultures have proven to be a versatile research tool.

For certain applications, transient expression systems can contribute significantly to functional gene analysis. Toward this goal, we have identified conditions to achieve transient expression from constitutive and fiber-specific promoters in fibers developing in culture (H.J. Kim, M.Y. Williams, and B.A. Triplett, unpublished data). When ovules are transformed via particle bombardment prior to fiber development, expression of the β-glucuronidase (GUS) reporter gene is evident within a few hours after transformation compared with a non-transformed control (Fig. 1, B and C). Fiber cells continue to develop after biolistic transformation and continue express the reporter gene (Fig. 1D). *Agrobacterium*-mediated transformation of cotton ovule cultures has also been reported (Delmer and Holland, 2000). As these techniques become more widely used, we propose that transient expression in cotton fiber will be a useful model for testing the function and regulation of genes associated with plant cell elongation.

LIMITATIONS OF THE COTTON FIBER MODEL SYSTEM

Despite many advantages that cotton fiber offers as a model for studying plant cell expansion and wall biogenesis, there are some limitations. Ovules in culture float on the surface of the liquid medium and produce copious amounts of fiber on the surface exposed to air. Unlike cell suspension cultures such as BY2 cells, these fibers are not fully submerged in the medium. Movement of nutrients, inhibitors, phytohormones, and other compounds must be controlled through uptake by the ovule. While this limitation mimics the way fibers develop in planta, it prevents assessment of rapid or short-term responses to exogenously added compounds. Recently, modified culture conditions were described in which the proportion of fibers growing on the submerged side of ovules was increased, however the morphology of submerged fibers was different from aerial fibers produced in vitro (Feng and Brown, 2000). A cell suspension culture from immature cotton ovules will elongate in response to gibberellin; however, unlike BY2 cells, the cotton suspension culture and ovular callus cells lose their capacity to elongate with prolonged culture periods (Trolinder et al., 1987).

The power of combining genetics with molecular and biochemical approaches is ably demonstrated by the stunning advances made in plant biology resulting from the Arabidopsis Genome Initiative. Although several cotton fiber developmental mutants are known, the limited number and difficulty in generating additional mutants is troublesome. Although the genus *Gossypium* has both diploid and tetraploid species, the commercially important varieties grown

in the United States are allotetraploids. The diploid species are difficult to grow both under field conditions in a temperate climate and in the greenhouse. Flower production is limited and the diploid plants often have a trailing habit. Due to the large genome size [estimated to be 12,642 Mbp (4C) (Bennett and Smith, 1976)], gene knock-out strategies so elegantly used in Arabidopsis (Krysan et al., 1999) are unsuitable for the functional analysis of cotton genes from the commercially important species. New strategies will need to be devised to selectively inactivate genes as a test for functionality.

Another limitation to the widespread use of this model system is that transformation and regeneration are slow and inefficient processes in cotton. Typical regeneration times can be as long as 6 to18 months with transformation frequencies ranging from 5% to 10% for *Agrobacterium*-mediated transformation to below 0.6% for biolistic transformation (McCabe and Martinell, 1993). One obsolete cultivar, *G. hirsutum* cv Coker 312, has the highest regeneration potential, whereas most commercially important elite varieties are difficult to regenerate. Various strategies for overcoming this regeneration barrier are being investigated including pollen transformation (Burke et al., 1997), floral transformation (Song et al., 1997), and shoot apex transformation (Gould and Magallanes-Cedeno, 1998).

NEW DIRECTIONS

The ease and speed of Arabidopsis transformation has been under-utilized by investigators to examine the expression patterns of cotton fiber promoter sequences and reporter genes. For example when Arabidopsis was transformed with a construct containing a cotton cellulose synthase gene promoter (CesA4, GenBank accession no. AF413210) fused to the GUS reporter gene, tissue-specific expression resulted. During fiber development, CesA4 is expressed only during the secondary wall-thickening stage (H.J. Kim, M.Y. Williams, and B.A. Triplett, unpublished data). In young Arabidopsis seedlings, GUS expression occurred in root tissue (Fig. 2A). At later stages of development, expression of the reporter was also evident in floral styles (Fig. 2B), at the tip and base of siliques (Fig. 2C), and in leaf vascular tissue (Fig. 2D). On closer inspection, the CesA4 promoter was active in Arabidopsis trichomes, but only in the basal region (Fig. 2E) and in stomatal guard cells on the leaf surface (Fig. 2F). We anticipate that continued analysis of cotton fiber promoters in Arabidopsis will accelerate the identification of promoter motifs that merit more detailed analysis in stably transformed cotton.

In spite of the difficulties with cotton transformation, cotton is one of the world's leading transgenic crops with over 12% of the land used for cotton production planted with transgenic varieties in 2001.

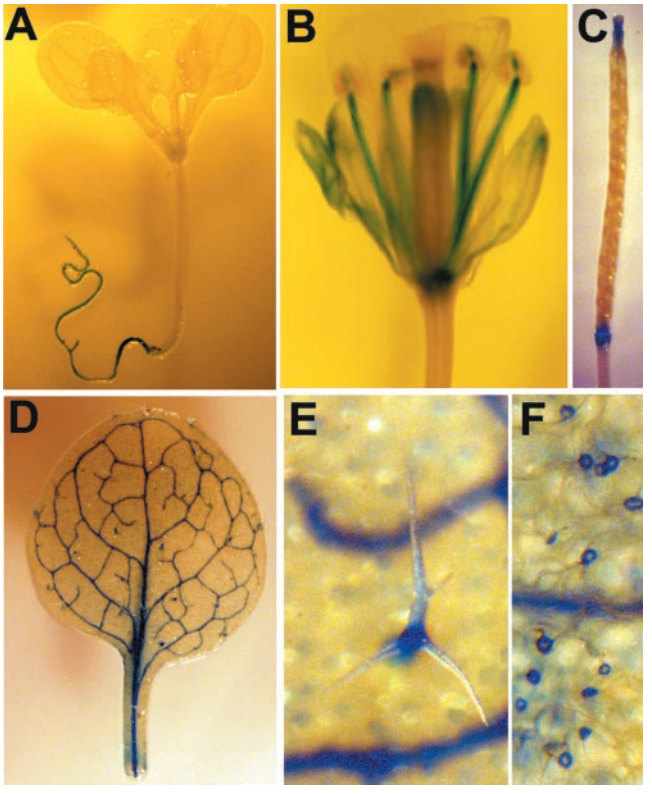

Figure 2. Histochemical GUS staining pattern of transgenic Arabidopsis transformed with a cotton fiber CesA4 promoter:GUS fusion in young seedling (A); inflorescence (B); mature silique (C); leaf (D); leaf trichome (E); and leaf surface showing stomatal guard cells (F).

Commercial varieties of transgenic cotton expressing the *Bacillus thuringensis* endotoxin gene (Bt) were first released in 1996. Herbicide-resistant cotton varieties followed in 1997 and soon thereafter varieties "stacked" with both the Bt and RoundUp Ready genes became available. Notwithstanding the relative importance of cotton as an important textile fiber, genome-mapping efforts have lagged behind other commodities. This delay may soon be reversed. In June 2001, a group of scientists representing many of the cotton-producing nations met in Montpellier, France, agreeing to increase communication, limit redundancy, and foster accelerated progress toward characterizing the cotton genome for the benefit of the public sector. This unprecedented effort, called the International Cotton Genome Initiative (http://algodon.tamu.edu/icgi/icgi.html), will bring a new level of organization to the public efforts of mapping the cotton genome.

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