PROGRESS IN GENETIC ENGINEERING OF COTTON FOR FIBER MODIFICATIONS

M. E. JOHN

Agracetus, Inc., 8520 University Green, Middleton, WI 53562, USA

Abstract

Modification of cotton fiber qualities through genetic engineering is the primary focus of the cotton biotechnology program at Agracetus, and the methodologies necessary to accomplish this task have been developed. A cultivar independent *(Accell®)** transformation method based on particle bombardment has been refined, and transgenic Delta and Pine 50/90, Pima, Sea Island, and Acala varieties have been generated. Stable integration of the transgene is shown in the parent and in subsequent progenies. A transient assay to detect promoter functions using particle bombardment and the marker gene ß-glucuronidase (*GUS*) has been developed. A number of cotton gene promoters have been isolated and tested in transgenic plants for their ability to direct fiber-specific gene expression. Genes from a variety of sources are currently being screened for their ability to modify fiber properties such as strength and length. In addition, we are assessing various genes for their ability enhance properties such as superior dye binding, absorbency, and thermal properties in cotton fiber. The resulting high-value transgenic fibers are expected to have a diverse impact on the fiber industry.

Introduction

Cotton, a worldwide crop valued at $20 billion, is the premiere natural fiber for textiles. Over the years conventional breeding programs have been remarkably successful in improving the fiber qualities and yield of cotton (Meredith, 1992; Sasser and Watson, 1992). Yet, the demands for still higher quality fibers and more cost effective cultivation, production, and processing practices require application of additional technologies that can achieve these goals in shorter time frames. Insect and herbicide resistant transgenic cotton generated through recombinant DNA methods is an example of the application of such a technology (Perlak *et al.*, 1990; Bayley *et al.*, 1992; Thomas and Bohnert, 1993). It also illustrates that traits found in bacteria or other sources can be incorporated into cotton. Thus, we now have the opportunity to produce fibers with new and novel properties to increase the product spectrum of fibers. What are the physical and chemical properties that can be targeted for modification in fiber? Properties such as strength and length that are strongly influenced by the genetics of the plant are prime targets. Other properties such as micronaire which denotes the fiber fineness (linear density) and maturity (wall thickness) that are mostly influenced by environmental factors may be more complex and difficult to address. In addition, fibers with superior dye binding abilities are also a major target. Table 1 shows examples of fiber qualities that may be altered through genetic engineering.

Our short term objective is to screen genes from a variety of sources, including cotton, that may have the potential to enhance fiber qualities. There are no model systems that can be used to test the effect of genes on fiber properties. Hence, each potential gene is being introduced into cotton and the transgenic fiber evaluated. This paper describes the necessary technology and progress towards this goal. A number of components are required for genetic engineering of cotton for fiber modification. The most crucial requirement is a diverse gene

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* *Accell®* is the trademark of Agracetus game delivery technology.

portfolio. Thousands of genes have been characterized from bacteria, fungi, plants and animals. Hopefully several of these genes will have potential for fiber modifications. However to determine their potential, they need to be modified and expressed in fiber. The following sections discuss the fiber-specific promoters and transformation technology required to integrate the genes into cotton genome.

Isolation and characterisation of cotton promoters

Genes that are predominantly expressed in fiber are the source of promoters to express various heterologous proteins in a fiber-specific manner. In order to isolate these genes, first the corresponding mRNAs are identified as follows.

Recombinant DNA libraries were constructed from mRNAs of 10-, 15-, 24-, and 28-day old fibers. These libraries were screened using cDNAs synthesized from RNAs of leaf, 0-day ovules, 10- 15- and 23-day old fibers, petals, and roots. A number of cDNA clones that hybridized strongly to the cDNAs corresponding to the fiber and not to other tissue RNAs were identified. These cDNA clones were further analyzed by northern blots to confirm their fiber-specific expression. For example when a northern blot containing RNA from leaf, 0-day ovule, petal, fiber, and root was hybridized to a fiber specific cDNA clone only RNA from fiber showed hybridization. Fiber specific cDNA clones were then used as probes to screen genomic libraries of *Gossypium hirsutum,* cv. C312 and *G. barbadense*, cv. Sea Island. Phages hybridizing to fiber specific cDNA clones were isolated and characterized by Southern blots and sequence analysis. Once the genes are isolated, their promoters can be identified by a transient marker gene expression system.

Particle bombardment can be used to identify the promoters of genes (John *et al.*, 1994). A Transcriptional fusion gene is generated by fusing the putative promoter to a promoter-less *GUS* gene. The plasmid construct is them introduced into cotton seed axes by particle bombardment. The cells take up the DNA and transcription occurs from any genes with a functional promoter within minutes or hours. This is demonstrated by introducing viral promoters that are functional in plants such as cauliflower mosaic 35s, or the promoter of the *Agrobacterium* gene nopaline synthase (Nos) that are ligated to a promoter-less *GUS* gene (Fig. 1). Plasmids without promoters on the other hand do not direct synthesis of the GUS enzyme. Uncharacterized DNA fragments containing promoter elements can be similarly introduced as chimeric *GUS* constructs and enzyme activity is detected by histochemical staining or fluorometric assay (Fig. 1). This procedure, while enabling one to assess promoter activity in a DNA fragment, does not address the question of tissue specificity. Generation of stable transgenic plants is required to assess tissue specific expression. Cotton promoters *FbE6-3B, FbE6-2A, Fb-H6*, and *Fb-B8* have been introduced into transgenic plants. In each instance, we have observed fiber-specific expression from these promoters (John and Crow, 1992; John, unpublished data).

Particle mediated transformation of cotton

The particle mediated gene delivery system of is the only technology to date shown to be capable of cultivar independent transformation of cotton (Umbeck *et al.*, 1987; McCabe and Martinell, 1993). Cultivar independence, the ability to introduce many genes into the same plant, time advantages over *Agrobacterium* mediated transformation, and the ability to produce epidermal or germline transformants are some of the salient points of this technology (Table 2). Epidermal and germline plants arise depending on which cells in the meristem received particles carrying DNA.

Epidermal transformants are transgenic plants where only the epidermal layer of cells carry the transgene. Thus, the progeny of these plants do not inherit the inserted gene.

However the epidermal transgenic plant (parent) can be used to assess the effect of transgenes on fiber modification, since the fiber originates from the epidermal layer. Due to the ease with which epidermal transformants can be produced, it is a preferred method for screening genes for fiber modification. Transformation of the progenitor cell for the germline in the meristem will result in a germline transgenic plant. Table 3 shows typical transformation frequencies of the epidermal or germline transformants generated in three independent experiments. As seen from this table, 59 to 80% of the total transgenics produced are epidermal. The progeny of germline plants carry the transgene and follow Mendelian inheritance. In the majority of the transgenics we have examined, only a few copies of the transgene are present (McCabe and Martinell, 1993). However, in a few cases we have observed more than a hundred copies. The gene copy number does not appear to be related to the expression level (data not shown).

Screening of genes for fiber modification

Of the many hundreds of potential genes available from various sources only few may have actual value in fiber modification. Genes that influence fiber initiation and development are a case in point. Fiber initiation and development is known to be influenced by plant growth regulators (Beasley, 1973; Beasley and Ting, 1973; DeLanghe *et al.*, 1978). Thus, perturbing the hormone balance in the fibers may offer an opportunity for fiber modification (Bhardwaj and Verma, 1985). The majority of known genes encoding enzymes involved in the biosynthesis of plant hormones are of bacterial origin (Morris, 1987). Examples are the *iaaH* and *iaaM* genes of the *Agrobacterium tumefaciens* pathway of IAA production, the *ipt* gene product that mediates synthesis of iPA-P from isopentenyl pyrophosphate and AMP, and the *iaaL* gene from *Pseudomonas savastanoi* that mediates the conjugation of IAA to Lysine (Barry *et al.*, 1984; Klee *et al.*, 1984). Plant hormones such as, auxins (IAA), gibberellims, ethylene, and abscisic acid have effects on the fiber development and properties (Kosmidou-Dimitropoulou, 1986). It is likely that there is a fine balance between the various hormones in fiber. We measured the free indole acetic acid (IAA) content in the fiber of three cultivars during their development and the results are shown in Figure 2. The IAA levels in both Pima and Sea Island fibers are much higher than that of DP50 during the 10- to 25-day of development. It is not known whether the higher fiber quality (strength, length or micronaire) of Pima and Sea Island in comparison to DP50 has any correlation with the IAA content. We are investigating the effect of perturbing the IAA levels in DP50 fibers by introducing genes *iaaH* and *iaaM* for the biosynthesis of IAA from tryptophan.

The *A. tumefacien* gene *iaaM* encodes tryptophan monooxygenase that converts trytophan to indole-3-acetamide (IAH). This intermediate is then converted to indoleacetic acid (IAA) by the enzyme encoded by *iaaH*, the indoleacetamide hydrolase. The coding regions of *iaaH* and *iaaM* genes along with their 3' untranslated regions were inserted into a vector cassette that contained fiber-specific promoter *FbE6-3B*, and a nopaline synthase promoter-*GUS* marker gene. This cassette, when introduced into cotton, directs the transcription of *iaaH* and *iaaM* genes in a fiber-specific manner. The marker gene *GUS* is expressed at low levels throughout the plant. A number of germline and epidermal transgenic DP50 plants were generated and their fibers are being tested. We measured the IAA content of 10- 15- and 20-day old fibers from one epidermal transgenic plant containing *iaaH* and *iaaM* genes and the results are shown in Table 4. The free IAA content in 15-day old fiber of #5492 shows an 8-fold increase over control DP50 fibers. These results show that we are able to introduce genes into cotton and express them in fiber. Characterization of mature fibers from #5792, as well as the remaining transformants and their progenies will enable us to assess the effect of perturbing of IAA levels on the length and strength of the fiber.

In summary, our technical strategy depends on the ability to screen potential genes that

affect fiber qualities. Necessary methodologies have been put in place for this task. Particle bombardment has been shown to be an efficient transformation methodology able to introduce multiple genes into upland as well as *barbadense* type cotton. Fiber promoters have been isolated and are shown to direct fiber-specific expression in transgenic plants. At the present time, we are screening a number of genes from various sources for their ability to modify cotton, and expect to identify a few that have real potential in the coming years.

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Table 1. Potential fiber property modifications by recombinant DNA technology.

Properties	Applications
Length, Strength[1]	Textiles.
Absorption[2]	Industrial and household absorbent; personal care products; disposable diapers. Medical applications.
Thermal Adaptability[3]	Specialty textiles; winter clothing.
Dye Binding[4]	Textiles.
Immobilization[5]	Food processing; medical, analytical application; bioremediation.
Reactive Groups[6]	Medical and industrial applications. Textiles.
Surface Modification[7]	Textiles; nonwovens.

Footnotes:

1. The normal range of fiber length varies for different cultivars. They range from 1 to 1.15 inches in upland varieties, up to 1.5 inches in *barbadense* types. Similarly, the upland varieties have a range of 23 to 33 gms/tex (HVI), whereas the *barbadense* type range from 30 to 40 gm/tex. The fiber strength is defined as the force required to break a bundle of fibers. The breaking load is converted to grams force per tex unit, where tex unit represents the weight in grams of 1000 meters of fiber.

2. The absorbency characteristics of cotton fiber are related to, surface properties, crystalinity, microporosity, and presence of hydroxly groups. Both increased or decreased water absorption properties of cotton have practical applications.

3. The ability to absorb and retain heat is important for winter wear. Cotton fiber has poor thermal properties; therefore enhancement of thermal properties therefore will create new applications.

4. Enhancement of dye binding properties of cotton must address higher dye yields and waste water toxicity, since large amounts of electrolytes are required to exhaust dye onto cotton. In addition, reactivity towards new dye compounds would be desirable.

5. Immobilizing enzymes within cotton fiber lumen or secondary walls will create a fiber capable of enzymatic reactions. Such fibers can be used in toxic clean up or industrial applications.

6. The major reactive group in cellulose is hydroxyl group. Presence of additional reactive groups within the secondary wall will increase its reactivity towards chemicals.

7. The surface properties of fiber affects its processing, as well as product properties. Modifications in the cuticle, wax components, and primary wall will affect both physical and chemical properties of fiber

Table 2. Characteristics of *Accell*® mediated cotton transformation.

Issue	Results
Cultivar independent	Transformed DP50/90; Pima; Sea Island; Acala; C312.
No tissue culture involved	No somoclonal variations. Growth and morphology of transgenic plants are normal.
Insertion of multiple genes	Up to 4 genes inserted.
Time savings	Savings of 6 months compared to *Agrobacterium* method.
Transformation frequency	0.2 to 0.7% based on explant survival.
Epidermal or germline	Epidermal plants are useful for gene screen.

Table 3. Cotton transformation frequencies.

Experiment	Explants Bombarded	Survived (%)	Total Transgenics (%)	Epidermal	Vascular
#1	13,292	6,475 (49%)	33 (0.51%)	24 (73%)	9 (27%)
#2	10,913	6,057 (56%)	45 (0.74%)	36 (80%)	9 (20%)
#3	12,278	5,368 (44%)	17 (0.32%)	10 (59%)	7 (41%)

Footnote: % Transformation is based on number of survived explants.
% Epidermal is based on total member of transgenics.
% Vascular is based on total number of transgenics.

Table 4. Free IAA content of transgenic #5492 fiber.

Cultivar	Free IAA content (ng/gm/FW)			
	10-day	15-day	20-day	25-day
DP50	10.7	12.1	25.2	6.7
#5492 (Transgenic DP50)	21.9	96.4	39.2	28.5
Pima	12.1	58.5	39.0	53.1

Figure 1. Transient *GUS* assay for the determination of promoter activity.

Mature cotton seeds were surface sterilized and incubated at 15˚C overnight in the dark to germinate. The radicle of the imbibed seeds were exposed, and seed axis removed. Ten cotton seed axes were bombarded (12 KV arc; DNA load rate of 1 ug/mg gold bead, 1-3 um) with various plasmids and left at room temperature in the dark on the agar plates for 24 hours to recover. Aliquots of 5 seed axes were then homogenized in 50 mM $NaHPO_4$, pH 7.0; 10 mM EDTA; 01.% sodium lauryl sarcosine, and 10 mM ß-mercaptoethanol. The supernatant of the homogenate was then mixed with MUG and fluorescence was measured after 60 min at room temperature (excitation at 365 nm, emission at 455 nm). The fluorometer was calibrated with freshly prepared 4-methyl umbelliferone (MU) standards. Plasmids *p35s-GUS* and *pNos-GUS* contain promoters from cauliflower mosaic virus and *Agrobacterium* nopaline synthase gene respectively. Plasmids *pFbE6-GUS*, *pFbB6-GUS*, and *pFbH6-GUS* contain promoters from cotton genes. *p2117* is a promoter-less GUS gene. *pF6-E6, pFb-B6*, and *pFb-H6* are cotton genes and do not contain any *GUS* marker genes. GUS activity shown are average value of duplicate experiments.

Figure 2. Free IAA content during fiber development.

Free IAA was measured after purification by solvent partitioning and high performance liquid chromatography. DP50, Pima, and Sea Island fibers (10-, 15-, 20-, and 25-day) or ovules with fiber (0-, 3-, and 5-day) were frozen in liquid nitrogen and subjected to IAA measurement after the addition of internal standards as described by Li et al., 1992. Results shown are the average value of two or more measurements for each time point. Additional measurements are being conducted to confirm these values.