



Commentary

A Novel Expression Assay System for Fiber-Specific Promoters in Developing Cotton Fibers

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Abstract. Cotton fibers are unicellular trichomes that differentiate from epidermal cells of developing cotton ovules. Cotton fibers are not only an economically important commodity, but they also are a good experimental model for studying plant cell elongation and wall biogenesis because of their fast elongation rate and high cellulose content. During fiber development, many genes are known to be transcriptionally regulated; however, because of the long periods required to regenerate transgenic cottons and the lack of suitable transient assay systems, progress in characterizing cotton fiber-specific promoters has been slow. We report an expression assay system for determining promoter activities in cotton fibers by using cotton ovule culture and biolistic transformation techniques. The conditions for bombardment into cotton ovules were optimized to enhance the transformation efficiency of the β -glucuronidase gene (*GUS*) into fiber cells. *GUS* gene expression patterns, which are regulated by a developmentally regulated fiber-specific promoter in cultured ovules, were consistent with the levels of the transcripts *in planta*. As a result, we propose that this newly developed expression assay system in cultured cotton fiber can be used to identify *cis* elements involved with the developmental and hormonal regulation of fiber development and fiber-specific expression.

Key words: biolistic transformation, cotton fiber, cotton ovule culture, *GUS* promoter activity assay, transient assay

Abbreviations: DOA, day of anthesis; DPA, days postanthesis; *GUS*, β -glucuronidase.

Introduction

Cotton is an economically important commodity worldwide. Cotton produces the world's leading natural fiber and the sixth most abundantly used oilseed. Because the fiber represents more than 90% of the total value of the cotton crop, the genetic improvement of fiber yield and quality is a major target for cotton biotechnology. Because of their highly elongated structure, high cellulose content, and lack of cell division, cotton fibers also are a good experimental model for studying plant cell elongation and cell wall biogenesis (Kim and Triplett, 2001). As a first step in the improvement of cotton fiber quality, many groups have isolated

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developmentally regulated genes from fibers, which may play important roles in determining key fiber properties. Genetic improvement of cotton fiber quality also requires the discovery of strong promoters specifically expressed in fiber. Several fiber-specific promoter sequences have been reported (Stalker et al., 1997; Hsu et al., 1999; Liu et al., 2000). Construction of stably transformed cottons with tissue-specific promoters is the best way to demonstrate the pattern of tissue-specific expression (Rinehart et al., 1996; Song et al., 2000). *Agrobacterium*-mediated and biolistic-transformation methods both have been used to construct transgenic cottons. However, recovery of transgenic plants requires many months of labor-intensive tissue culture (Chlan et al., 1995; Wilkins et al., 2000). As a result, progress in characterizing cotton fiber-specific promoters has been much slower than that in other plants, and comparatively little is known about *cis* elements in fiber promoters.

To circumvent the difficulties of regenerating cotton, fiber-specific promoters have been studied in tobacco (Hsu et al., 1999; Liu et al., 2000) and *Arabidopsis* (Kim and Triplett, 2001). These heterologous systems were tested to determine if they conferred leaf trichome-specific expression. Promoter analyses in tobacco, transformed with 2 cotton fiber lipid transfer protein promoters, showed that relatively short promoters of fewer than 614 nucleotides were enough to confer trichome-specific expression in tobacco leaves (Hsu et al., 1999; Liu et al., 2000). When a promoter from the cotton fiber *CesA* family (catalytic subunit of cellulose synthase) was introduced into *Arabidopsis*, reporter expression occurred in leaf trichome cells and other tissues (Kim and Triplett, 2001). However, promoter analyses of cotton fiber-specific promoters with heterologous systems cannot be expected to completely identify the promoter *cis* elements that are required for tissue-specific or temporal expression.

To develop a quick and simple expression assay for determining promoter activities in developing cotton fibers, we have transformed cotton ovule cultures biolistically. A culture method for producing fiber on developing cotton ovules has been used by many investigators (Beasley and Ting, 1973). With this culture system, we have optimized conditions for particle bombardment with the GUS reporter and assayed a fiber-specific promoter in the optimized expression assay system. From this study, we propose that the expression assay system in cultured cotton ovules may provide a much quicker and simpler way to examine fiber-specific expression and developmental/hormonal regulation than constructing transgenic cotton plants.

Materials and Methods

Plant material

Plant materials for RNA isolation were harvested from field-grown *Gossypium hirsutum* var. DPL90. Developing bolls were harvested at 2-day intervals from 10-20 DPA. The fibers were carefully removed from the ovules for RNA extraction. DOA ovules were used for genomic DNA extraction. Ovule cultures were initiated with either DOA or 2 DPA ovules from greenhouse-grown plants.

DNA constructs

Constructs supplied by other laboratories were used without modification (Table 1). The construct, p*GhCesA4*:*GUS*, was constructed in this lab by transferring the 2.6-kb promoter of *GhCesA4* (*Sal* I / *Eco*R I) into the polylinker site of a pCAMBIA1391z vector (Roberts et al., 1998) (Table 1).

Cotton ovule culture and particle bombardment

Bolls from the fiber initiation stage (DOA to 2 DPA) were harvested, surface-sterilized in 95% ethanol, flamed briefly, and dissected under sterile conditions. Ovules were transferred to a liquid culture medium (BT medium) in the presence of 5 μ M indole-3-acetic acid and 0.5 μ M gibberellic acid (Beasley and Ting, 1973). The ovules were placed on 2 Whatman filter papers premoistened with BT medium, a solid BT medium containing 0.8% agar, or a solid BT medium containing 0.15% PhytigelTM (Triplett and Johnson, 1999) for bombardment. DNA was precipitated onto 1- μ m gold particles by the calcium chloride–spermidine method described in the Bio-Rad (Richmond, CA) instruction manual for the Biolistic Particle Delivery System (1000/He). Cultured ovules were bombarded with a constant 9-cm target distance in 28-inch Hg vacuum according to the manufacturer's recommendation for plant tissues and with various Helium pressures (450, 1100, 1350, 1550, and 2000 psi). The bombarded ovules were transferred immediately to a fresh liquid BT medium or were incubated on a solid BT medium containing 0.15% PhytigelTM (Triplett and Johnson, 1999) for 2 d before transfer to a liquid BT medium. A single layer of Micropore surgical tape (3M Healthcare, St. Paul, MN) was wrapped around each dish. Cultures were grown at 32°C in a 5% CO₂ atmosphere. Each construct was bombarded into more than 120 ovules per replicate and was repeated more than 6 times.

Analysis of β -glucuronidase expression patterns

Histochemical localization of GUS enzyme activity was carried out using 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) as described by Jefferson (1987). To prevent diffusion of the GUS product during staining, 0.5 mM of potassium ferri/ferrocyanide was added to the histochemical staining buffer. Pictures of histochemically stained ovules associated with fibers were taken with an Olympus SZX stereomicroscope with an Olympus DP11 digital camera. Image composites were constructed using Adobe Photoshop 5.5 software.

Cloning of GhCesA4, a gene expressed during the secondary cell wall thickening stage

Differential display (Liang and Pardee, 1992) was used to isolate genes that are expressed concomitantly with secondary wall thickening. Fiber cDNAs were synthesized from total RNAs isolated from the elongation stage (10 DPA) and the secondary wall thickening stage (20 DPA) using oligo-dT primers and MMLV reverse transcriptase. Polymerase chain reaction amplification in the presence of 20 different arbitrary 5' primers and oligo-dT 3' primers (Liang and Pardee, 1992) was conducted as described by the manufacturer (RNAimage kit; GenHunter, Nashville, TN). The [³³P]-dATP-labeled amplified cDNA subpopulation from

Table 1. Constructs used in biolistic bombardment. TL, tobacco mosaic virus leader sequences.

Constructs	Promoter:reporter	Reference
pCAMBIA 1301	CaMV35S:GUS	Roberts et al., 1998
pAGUS1-TN2	double CaMV35S:TL:GUS	Skuzeski et al., 1990
pGhCesA4:GUS	Cotton cellulose synthase A4 promoter:GUS	Kim and Triplett, 2001

each primer pair was separated on a 6% denaturing polyacrylamide gel (45 cm). After electrophoresis, the gel was dried and exposed to X-OMAT AR film (Kodak, Rochester, NY). Amplification products that were more abundant in the 20 DPA lanes compared with products in the 10 DPA lanes were recovered from the dried gel. Recovered cDNAs were reamplified using the same primer set and PCR conditions as in the first amplifications. The expression patterns of all candidates were confirmed by northern blot analysis (e.g., Figure 2), and the complete nucleotide sequences were determined. One of the differential display products shared some sequence similarity to *CesA1*, a putative catalytic subunit of cellulose synthase (Pear et al., 1996). Because of sequence divergence between *CesA1* and the gene discovered by differential display, we have named this gene *CesA4*.

Cotton genomic DNA was isolated from DOA ovules using Plant DNAzol (Invitrogen, Carlsbad, CA) and was used to construct a genomic library using Lambda FIX II / *Xho* I partial fill-in vector kit (Stratagene, La Jolla, CA). A full-length genomic clone corresponding to *CesA4* and its corresponding promoter of 2.6 kb (GenBank accession number, AF413210) was isolated from the genomic library by using a radiolabeled *GhCesA4* cDNA as a probe.

RNA isolation and analysis

Total RNA was extracted from cotton fibers at different developmental stages by phenol extraction, LiCl precipitation (Schultz et al., 1994), and final purification with a kit (RNeasy Kit, Qiagen, Valencia, CA). Three micrograms of total RNA were separated on 1.2% agarose gels containing formaldehyde (Lehrach et al., 1977). The RNA was transferred onto BrightStar-Plus nylon membranes (Ambion, Austin, TX) with 5X SSC and 10 mM NaOH. *GhCesA4* RNA probe was labeled with [³²P]-UTP (3000 Ci / mmol) by in vitro transcription (Ambion, Austin, TX) using the SP6 promoter. The membranes were hybridized with the radiolabeled RNA probe (>10⁹ cpm/μg specific activity), washed at 65°C in 0.1 X SSC and 0.1% SDS, and autoradiographed.

Results

GUS expression in cotton fibers

To test if *GUS* is expressed in cultured ovules, two *GUS* constructs were used (Table 1). A binary vector, pCAMBIA 1301, contains *GUS* regulated by an 800-nucleotide CaMV 35S promoter (Roberts et al., 1998). Another vector, pAGUS1-TN2, contains *GUS* regulated by two repeated copies of 300 nucleotides from the CaMV 35S promoter fused to the tobacco mosaic virus leader sequence (TL) (Skuzeski et al., 1990). Histochemical staining of unbombarded 2 DPA ovules

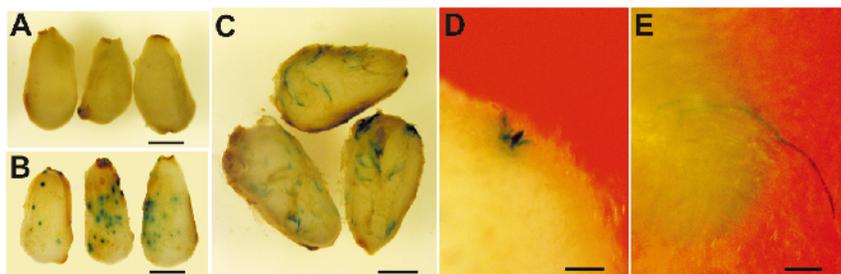


Figure 1. Histochemical assays for *GUS* expression in cultured cottons bombarded by *pAGUS1-TN2*. Histochemical localization of *GUS* enzyme activity was carried out using 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) as described by Jefferson (1987). Histochemical staining of (A) unbombarded 2 DPA ovules, (B) 2 DPA ovules bombarded on DOA, (C) 3 DPA ovules bombarded on 2 DPA, (D) initiating fibers of 2 DPA ovule, (E) elongating fibers of 8 DPA ovule. Scale bar in A, B, C, and E = 500 μ m, D = 200 μ m.

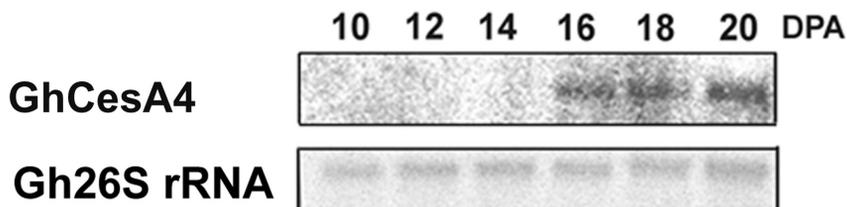


Figure 2. Developmental expression of *GhCesA4*. Total RNA was isolated from 10–20 DPA fibers. Three micrograms of total RNA were separated on 1.2% agarose gels containing formaldehyde. The RNA of gels was transferred onto positively charged nylon membranes. The membranes were hybridized to [32 P]-labeled *GhCesA4* antisense riboprobe and washed in 0.1X SSC and 0.1% SDS at 65°C. Ribosomal *Gh26S* RNA was used to quantify differences in RNA loading.

(Figure 1A) showed no *GUS*-like activity that has been reported previously in some plants, particularly in the seeds (Hu et al., 1990). The DOA ovules bombarded with *pAGUS1-TN2* and stained on 2 DPA showed a strong *GUS* expression in epidermal tissue (Figure 1B). *GUS* expression also was evident in developing fibers (Figures 1C, 1D, and 1E).

Optimization of conditions for particle bombardment

Because *pAGUS1-TN2*, a small vector containing viral leader sequences (Skuzeski et al., 1990), showed stronger *GUS* expression than *pCAMBIA 1301*, conditions for particle bombardment to DOA cotton ovules were optimized with *pAGUS1-TN2*. Five different helium pressures (450, 1100, 1350, 1550, and 2000 psi) for bombardment were tested. Transformation efficiency of *GUS* was very low, with pressures lower than 1100 psi. When bombarded with more than 1550 psi, most bombarded ovules on the liquid BT medium sank and were unable to produce

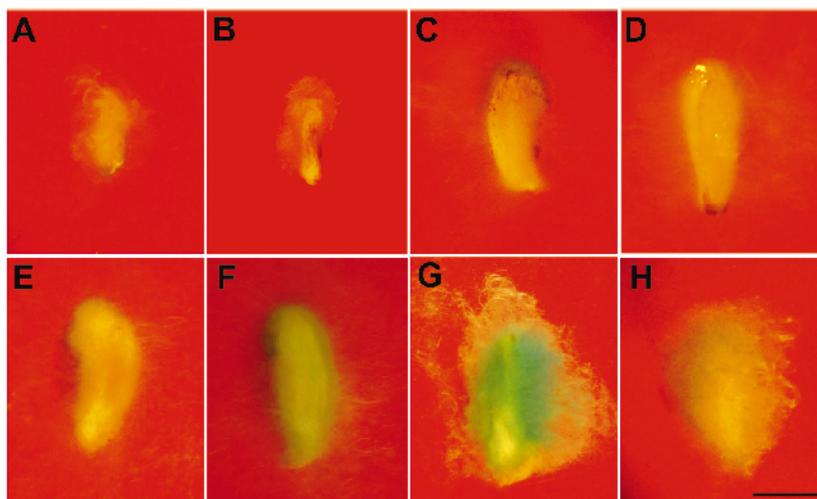


Figure 3. Histochemical assays of cultured cottons bombed by a developmentally regulated promoter *GhCesA4* fused with *GUS*. *pGhCesA4:GUS* was bombed into DOA cotton ovules and incubated with a BT liquid medium. Cultured ovules were harvested according to developmental stages and stained with X-Gluc. Histochemical staining of (A) unbombed 6 DPA ovule (control), (B) bombed 6 DPA ovule, (C) bombed 9 DPA ovule, (D) bombed 12 DPA ovule, (E) bombed 15 DPA ovule, (F) bombed 18 DPA ovule, (G) bombed 24 DPA ovule, and (H) unbombed 24 DPA ovule (control). Scale bar is 5 mm.

fibers. Thus, we chose 1350 psi as an optimal condition for bombardment onto DOA cotton ovules.

For bombardment, ovules were placed on Whatman filter paper pre-moistened with BT medium or on agar. When ovules were transferred immediately after bombardment to fresh liquid BT medium, a high percentage of the ovules sank and were unable to produce fiber. To increase the viability of bombed ovules, ovules were bombed on a solid BT medium containing 0.15% (wt/vol) Phytigel™ (Triplett and Johnson, 1999) and allowed to recover on the same solid medium for 2 d. After recovery, the bombed ovules were transferred to liquid BT medium. More than 95% of the recovered ovules from the solid BT medium floated on the liquid BT medium, developed normally, and produced fibers.

Histochemical staining of 2 DPA ovules bombed on the DOA showed that *GUS* mostly was found in epidermal cells, with only a few of them developing fibers later (Figure 1B). To increase the efficiency of *GUS* reporter expression in fiber cells on the cultured ovules, we compared transformation with *pAGUS1-TN2* on ovules in early (DOA) and late (2 DPA) fiber initiation stages. Histochemical staining of 3 DPA ovules bombed on 2 DPA showed that *GUS* activity mostly was found in fiber cells of bombed ovules (Figure 1C).

Cloning of a promoter associated with cotton fiber secondary wall thickening stage

A cDNA fragment, specifically expressed during the secondary wall thickening stage of cotton fiber development, was cloned by differential display (Liang and Pardee, 1992). DNA sequence analysis of the differential display product showed sequence homology to the cotton fiber CesA family (Pear et al., 1996), whose members function as the catalytic subunit for cellulose synthase. A genomic clone (*GhCesA4*) was obtained by screening a cotton genomic library using the differential display fragment as a probe. In addition to the coding sequence, a 2.6-kb promoter for *GhCesA4* also was obtained (GenBank accession no. AF413210).

Northern blot analysis shows that the transcript levels of *GhCesA4* are developmentally regulated in developing fiber cells *in planta* (Figure 2). Transcripts of *GhCesA4* barely were expressed during the elongation stage (10-14 DPA) and began to be expressed when the secondary wall thickening stage began (16 DPA). This pattern of expression is consistent with an earlier observation that the initiation of secondary cell wall biosynthesis in developing cotton fiber occurred from 14-16 DPA in this cotton variety (Whittaker and Triplett, 1999).

Expression assay of developmentally regulated cotton fiber-specific promoters in cultured cotton fibers

The promoter (2.6 kb) of *GhCesA4* was fused to the *GUS* reporter gene in CAMBIA vector 1391z (Roberts et al., 1998), in which a catalase intron was inserted in *GUS* to prevent any possible GUS activity from residual DNA constructs in the medium. Cultured ovules bombarded on 2 DPA by *pGhCesA4:GUS* were harvested after various periods postbombardment and were stained with X-Gluc for 16 h. Expression of *GUS* in cultured ovules regulated by the *GhCesA4* promoter rarely was detected at 6, 9, 12, and 15 DPA (fiber elongation stages) (Figures 3B, 3C, 3D, and 3E). Occasionally, very weak but detectable blue spots on bombarded ovules were observed by microscopic analysis during the elongation stage. As the secondary wall thickening stage of fiber development began, *GUS* expression dramatically increased so that entire ovules and fibers at 18 and 24 DPA were blue despite the presence of potassium ferri/ferrocyanide in the histochemical staining buffer to prevent diffusion of the GUS product (Figures 3F and 3G). Unbombarded 6 and 24 DPA ovules were used as negative controls (Figure 3A and 3H). The pattern of *GUS* gene expression controlled by the *GhCesA4* promoter in cultured ovules (Figure 3) was consistent with the developmental expression levels of *GhCesA4* transcripts *in planta* (Figure 2).

Discussion

In this study, we report the development of an expression assay system to analyze promoter activities expressed in cotton fiber by using cultured cotton ovules and biolistic transformation techniques. Many cotton fiber-specific genes are regulated transcriptionally during fiber development (John and Crow, 1992; Pear et al., 1996; Rinehart et al., 1996; Shimizu et al., 1997; Song and Allen, 1997; Smart et al., 1998; Shin and Brown, 1999; Whittaker and Triplett, 1999). However, the

identities of promoter *cis* elements that control cotton fiber developmental, hormonal, and tissue-specific gene expression remain unknown.

Having a transient assay for monitoring gene expression in fiber will provide many advantages over the long period that is required to produce stably transformed cotton plants. Over the last decade, transient assay systems using plant protoplasts, leaf tissue, and seeds have been employed to deduce the *cis* elements of many plant promoters. Because cotton fibers grow from the epidermal layer of cottonseeds, we have patterned our assay system after expression systems developed for seeds. For example, a transient assay system in barley aleurone was developed to identify *cis*- and *trans*-acting elements involved in the coordinated gene expression regulated by gibberellins and abscisic acid (Lanahan et al., 1992; Cercos et al., 1999; Gomez-Cadenas et al., 1999). In contrast to the relatively short periods of transient expression (24-48 h) in the barley aleurone, we wished to achieve much longer-term expression in the cotton ovule culture expression system. Longer and more stable expression was needed for monitoring fiber promoters from developmentally regulated genes that should be expressed either during cell elongation (<16 DPA in culture) or during secondary cell wall thickening stages (>14 DPA in culture).

For optimization of particle bombardment into cultured ovules, p*AGUS1-TN2* vector (Table 1) (Skuzeski et al., 1990) containing double CaMV 35S promoters and a viral leader sequence (TL) was used because it showed higher transformation efficiency than other GUS vectors, pCAMBIA 1301 (Roberts et al., 1998) and pBI221 (Clontech, Palo Alto, CA). Previously, it was reported that p*AGUS1-TN2* showed approximately 20 times higher GUS expression than pBI221 when it was expressed transiently in BY2 protoplasts (Skuzeski et al., 1990). *GUS* gene bombarded into cultured ovules was expressed in ovule epidermal cells 24 h after bombardment (Figure 1B) and stably expressed in cotton fibers 1 wk after bombardment (Figure 1E). For this reason, we chose *GUS* as a reporter for further optimization of particle bombardment into cultured cotton.

For high transformation efficiency of *GUS* with less damage to the cultured ovules, we first determined the optimal pressure for bombardment. Pressures higher than 1550 psi killed most ovules, and pressures lower than 1100 psi showed very low transformation efficiency. Although 1350 psi showed the best transformation efficiency and survival rate of bombarded ovules, a high percentage of bombarded ovules still sank in the liquid BT medium and did not produce fibers when ovules were transferred directly onto the liquid BT medium after bombardment. To address this problem, a recovery step was added, which allowed the bombarded ovules to remain on the same solid BT medium containing 0.15% (wt/vol) PhytigelTM (Triplett and Johnson, 1999) for 2 d after bombardment before being transferred to liquid BT medium. Most ovules remained on the surface of the liquid BT medium and continued fiber development.

Another problem we encountered was that most GUS expression appeared to be limited to the epidermal layer rather than fiber cells of the cultured ovules when DOA ovules were used for bombardment (Figure 1B). Fewer fibers had developed in the bombarded area of cultured ovules. One possible reason for this is that fiber initiation may be blocked by the physical damage associated with the particle bombardment. Therefore, we bombarded 2 DPA ovules on which fiber

initiation already had occurred and found that a greater degree of GUS was expressed in fiber cells (Figure 1C).

Although transient expression of GUS activity may be independent of the extent of cell division activity, such as in wheat (Goodling et al., 1999), its stable expression is more likely to occur in rapidly proliferating cells (An et al., 1988; Paszty and Lurquin, 1987; deKathen and Jacobsen, 1995; Villemont et al., 1997; Rajasekaran et al., 2000). Between 2 and 5 DPA, the nuclei of cotton fiber cells undergo continuous cycles of endoreduplication (van't Hof, 1999), thereby potentially improving the chance for stable integration of foreign DNA into fiber nuclei.

Because of the stability of GUS protein, it is difficult to assess if *GUS* expression in fibers 1 wk after bombardment by pAGUS1-TN2 (Figure 1E) came from newly translated GUS or resulted from the accumulation of GUS that was transiently expressed immediately after bombardment in fibers. Thus, we have addressed 2 additional issues to show that cotton ovule cultures are a suitable target for biolistic transformation. The first issue is how long reporter genes can be expressed after bombardment. The second issue is whether developmentally regulated promoters express the same patterns in cultured ovules as *in planta*. For this reason, we have isolated *GhCesA4* cDNA, a gene that is specifically expressed during the secondary wall thickening stage in developing cotton fibers (Figure 2). The complete gene of *GhCesA4* (accession number, AF413210) containing a 2.6-kb promoter was cloned from a cotton genomic library and showed a high sequence similarity with cellulose synthase A1 (*CesA1*) (Pear et al., 1996). Expression of *GUS* regulated by the *GhCesA4* promoter rarely was detected during the elongation stage in cultured ovules, but *GUS* expression dramatically increased as the secondary wall thickening stage of fiber development began (Figure 3). These results are consistent with previous observations that the rate of cellulose synthesis (Meinert and Delmer, 1977; Whittaker and Triplett, 1999) and the transcript levels of *CesA1* in cotton fibers (Pear et al., 1996) increased dramatically with the onset of the secondary wall thickening stage. The expression pattern of GUS controlled by the *GhCesA4* promoter in bombarded cultured ovules was the same developmental pattern as the accumulation of *CesA4* transcripts *in planta*. These results clearly show that the bombarded GUS reporter was expressed stably in fiber cells in the 2-3 weeks after bombardment, and that the levels of *GUS* expression in the bombarded cultured ovules was totally dependent on a developmentally regulated promoter.

We also tested if 2 other reporter genes, luciferase (*LUC*) and green fluorescent protein (*GFP*), are expressed in the expression assay system. *LUC* and *GFP* were chosen because they have been used extensively in higher plants, their endogenous activities are low, and sensitive assays are available. However, we were unable to detect *LUC* or *GFP* activity in developing cotton fiber cells in our expression assay system despite the use of improved *GFP* genes, including *mGFP5*, *mGFP5-ER* (Haseloff et al., 1997), and *smGFP* (Davis and Vierstra, 1998) fused with CaMV 35S. GFP activity also was detected rarely in bombarded cotton embryos (J. Cary, personal communication), but one report suggests that *GFP* was expressed stably in cotton cotyledon callus (Sunilkumar and Rathore, 2001). Therefore, we suspect that unknown environments in cotton cells might cause

insolubility, instability, or toxicity of GFP, resulting in their lack of expression in developing fiber cells as in some other plants (Haseloff et al., 1997; Davis and Vierstra, 1998).

Although the results in this study show that the expression assay with cultured ovules is a very useful technique to detect the promoter activities expressed in cotton fibers, the assay has several limits. The first limitation is caused by a characteristic of GUS, in that it has a relatively long half-life protein. The level of *GUS* expression during fiber development was regulated by a developmentally up-regulated promoter, such as *pGhCesA4* (Figure 3). On the other hand, it may be difficult to detect dramatic decreases in expression by promoters that are developmentally down regulated during fiber development because of the stability of GUS protein. The second limitation is caused by an inability to control transformation efficiency. The results of any promoter assay are affected by the transformation efficiency of bombardment and by promoter activities. Therefore, it may be necessary to normalize the transformation efficiency between different batches of bombardments with an internal standard when quantitative analyses of promoter activities are needed.

Despite these limitations, this newly developed expression assay system using cotton ovule cultures and biolistic transformation can be a useful tool to determine the promoter *cis* elements needed for fiber-specific expression and developmental or hormonal regulations without the need to produce transgenic cotton plants.

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