Fiber-specific genes are expressed preferentially or exclusively in cotton (*Gossypium* spp.) fiber and are thought to have important functions in fiber development. The promoters of these genes are of interest because they control transcription in the fiber cell and may be used in the genetic manipulation of fiber quality. The promoter of a cotton lipid transfer protein gene, *FSltip4*, was isolated and shown to direct fiber-specific transcription of an abundant mRNA in cotton. In transgenic tobacco, this promoter was strongly active in leaf trichomes. Deletion analysis of the promoter identified an AT-rich 84 bp fiber specificity region (FSR) necessary for activity exclusively in the fiber cells. Cotton fiber proteins that bind the FSR were isolated using a yeast one-hybrid assay. One of these was a putative AT-hook motif.

GhAT1 was shown to be nuclear localized, and GhAT1 transcripts were found to be preferentially expressed in ovules and non-fiber tissues. Overexpression of GhAT1 strongly repressed the activity of the *FSltip4* promoter in the trichomes of transgenic tobacco. These results suggest that GhAT1 assists in the specification of fiber cells by repressing *FSltip4* in the non-fiber tissues of the cotton plant.

**Keywords:** AT-Hook — Cotton — Fiber — Promoter — Trichome.

Abbreviations: AR, activation region; 3-AT, 3-aminotriazole; DPA, days post-anthesis; FSR, fiber specificity region; GFP, green fluorescent protein; GUS, β-glucuronidase; HMGA, high mobility group A; LTP, lipid transfer protein; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR.

The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers EF140831 (*FSltip4* gene and promoter) and EF140832 (*GhAT1* coding sequence).

**Introduction**

Cotton fibers are highly elongated single cells of the outer epidermal layer of the cotton ovule. Fiber development commences on the day of anthesis (flowering) and is divided into four distinct but overlapping phases: initiation, elongation, secondary wall synthesis and maturation (Basra and Malik 1984, Basra and Saha 1999). These phases are regulated by the ordered expression of several thousand genes in the fiber cell (John and Crow 1992, Arpat et al. 2004). A proportion of these genes are fiber specific (expressed preferentially or exclusively in the cotton fiber) and these are assumed to have important roles in fiber development. The promoters of fiber-specific genes may regulate gene function (and hence fiber development) by restricting transcription to the fiber cell. Several promoters have been shown to drive fiber-specific gene expression in transgenic cotton (Wilkins et al. 2000, Li et al. 2002, Li et al. 2005). Fiber characteristics have also been manipulated by coupling fiber-specific promoters to specific transgenes that may improve fiber quality (John and Keller 1996, Rinehart et al. 1996, John 1999).

Efforts to understand and manipulate fiber development would be enhanced by an improved knowledge of the promoter elements and transcription factors that regulate fiber-specific gene expression. However, promoter analysis in cotton is limited by the resources and extended timeline (minimum 12 months) required to generate transgenic plants (Wilkins et al. 2000). In contrast, the genetic control of trichome (epidermal hair) morphogenesis has been extensively characterized in *Arabidopsis thaliana* (Larkin et al. 2003, Pesch and Hulskamp 2004). Trichomes in *Arabidopsis* and tobacco (*Nicotiana tabacum*) show a number of structural and genetic similarities to cotton fibers (Wang et al. 2004, Humphries et al. 2005). Hence, it has been possible to monitor the activity of cotton fiber-specific promoters in these heterologous model species (Hsu et al. 1999, Liu et al. 2000, Wang et al. 2004, Wu et al. 2006).

Lipid transfer proteins (LTPs) are small basic proteins that are abundant in the epidermal tissues of many plants (Kader 1996). Their precise functions are unknown, but they are capable of transferring phospholipids between membranes in vitro and have been implicated in cutin deposition (Ma et al. 1995, Pyee and Kolattukudy 1995) and long-distance signaling in response to pathogens (Maldonado et al. 2002). A number of LTP genes are preferentially expressed in cotton fibers (Ma et al. 1995, Ma et al. 1997, Orford and Timmis 1997, Orford and...
GhExp1 demonstrated that the bombarded cotton leaves, petals and 3 DPA fibers to GUS staining as predicted. Similarly, Harmer et al. (2002) secondary wall synthesis (at 18 and 24 DPA) showed strong activity during secondary wall synthesis. Fibers undergoing by the promoter of \( \text{GhCesA4} \) anthesis (DPA) with a construct containing GUS regulated (2001) bombarded cotton fibers collected 2 days post-tissues are then allowed to recover and analyzed for tissues by microprojectile bombardment. The bombarded regulated by the promoter of interest are delivered to cotton alternative method for the analysis of fiber-specific promoter regions and cognate transcription factors necessary for trichome-specific gene expression. Deletions of the \( Ltp6 \) promoter resulted in a progressive reduction in GUS activity in the transgenic plants, and activity ceased with removal of the basal promoter elements (CAAT and TATA boxes) (Hsu et al. 1999). In contrast, deletion of the \( Ltp3 \) promoter identified a 315 bp region (–614/–299) necessary for trichome-specific GUS expression (Liu et al. 2000).

Transient assays of promoter activity provide an alternative method for the analysis of fiber-specific promoters. In this approach, constructs containing a reporter gene regulated by the promoter of interest are delivered to cotton tissues by microprojectile bombardment. The bombarded tissues are then allowed to recover and analyzed for transient expression of the reporter. Kim and Triplett (2001) bombarded cotton fibers collected 2 days post-anthesis (DPA) with a construct containing GUS regulated by the promoter of \( \text{GhCesA4} \) (a cellulose synthase gene active during secondary wall synthesis). Fibers undergoing secondary wall synthesis (at 18 and 24 DPA) showed strong GUS staining as predicted. Similarly, Harmer et al. (2002) bombarded cotton leaves, petals and 3 DPA fibers to demonstrate that the \( \text{GhExp1} \) expansin gene promoter is active only in fiber cells.

This report describes the isolation and functional analysis of the promoter of a fiber-specific gene (\( FSltp4 \)) to identify the promoter regions and cognate transcription factors necessary for fiber-specific gene expression. Deletion analysis of promoter activity in cotton fibers identified an 84 bp region necessary for confining gene expression to the cotton fiber. Transcription factors that interact with the 84 bp region were identified in a yeast one-hybrid assay, and one of these factors was further analyzed to determine its potential role in the regulation of fiber-specific gene expression.

**Results**

*Isolation and functional characterization of the FSltp4 promoter*

In previous work, we identified an LTP cDNA (pFS6) that is abundant in elongating cotton fibers between 6 and 14 DPA (Orford and Timmis 1997). Southern blot analysis suggested that allotetraploid cotton contained 6–8 LTP-encoding genes hybridizing to pFS6. Three of these were isolated, but none of them encoded the fiber-specific cDNA (Orford and Timmis 2000). A PCR-based genome-walking strategy was used to isolate the 1,888 bp gene and promoter sequences corresponding to pFS6 (Fig. 1; GenBank accession No. EF140831). The gene sequence contained a putative 80 bp intron near the 3’ end of the coding region flanked by conserved donor and acceptor splice sites, but was otherwise identical to the pFS6 sequence. The gene was designated \( FSltp4 \) in accordance with the nomenclature used in previous work in which \( FSltp1–FSltp3 \) were described (Orford and Timmis 2000). This designation also distinguishes the gene from previously described LTP genes, in particular \( Ltp3 \) (Liu et al. 2000) and \( Ltp6 \) (Hsu et al. 1999). The \( FSltp4 \) gene and promoter sequences demonstrate >90% nucleotide identity with the corresponding sequences in \( Ltp3 \). However, the mature polypeptide encoded by \( FSltp4 \) shows only 88% amino acid identity with that encoded by \( Ltp3 \). This suggests that \( FSltp4 \) and \( Ltp3 \) are allelic or alloallelic genes derived from a common ancestral sequence.

Microprojectile bombardment of cotton leaf, epicalyx, petal and 3–5 DPA fibers with an \( FSltp4::GUS \) reporter construct containing GUS regulated by the \( FSltp4 \) promoter produced GUS expression only in the fiber (Table 1). In situ hybridization of 0 DPA ovules with an antisense \( FSltp4 \) RNA probe produced strong staining in the fiber initials, but no staining was visible in the other cells of the ovule or in ovules probed with a control sense \( FSltp4 \) probe (Fig. 2A, B). These results are consistent with the fiber-specific expression pattern of the \( FSltp4 \) transcript (Orford and Timmis 1997), and demonstrate that the promoter is fiber-specific in cotton.

The high level of sequence similarity between the \( FSltp4 \) and \( Ltp3 \) promoters suggested that they would function in a similar manner in transgenic tobacco. Hence the activity of the \( FSltp4 \) promoter was tested by generating \( FSltp4::GUS \) tobacco. Four independent \( FSltp4::GUS \) lines were obtained, and histochemical staining demonstrated a similar pattern of GUS expression in each line. Strong GUS activity was detected in all types of trichomes, and expression was also visible at the leaf margin and in the vascular tissue (Fig. 2G–I). In the flowers, GUS expression was visible in the stigma, anthers, anther trichomes, and sepal and petal margins (Fig. 2J, K). No expression was detected in the pollen (data not shown), but most lines showed GUS activity in the ovules and capsule wall (Fig. 2L). In 21-day-old T2 transgenic seedlings, strong GUS expression was detected in the root tips, lateral roots, axillary shoot primordia, vascular tissue and emerging leaves (Fig. 2M–O). In contrast to the results of Sudan et al. (2006), no GUS expression was observed in non-transgenic control tobacco plants of similar age and tissue type.
The high level of GUS expression in the trichomes was similar to that observed by Liu et al. (2000), suggesting that the FSltp4 and Ltp3 promoters behave similarly in tobacco. However, staining in non-trichome tissues was not reported by Liu et al. (2000), preventing a more detailed comparison of the two promoters from the published literature. The GUS expression profile of the FSltp4::GUS plants was also consistent with the general characteristics of LTPs. A high level of GUS expression was detected in epidermal and mitotically active tissues (e.g. leaf margins) that require a high level of cutin production and hence LTP expression.

An 84 bp fiber-specificity region (FSR) is essential for gene expression exclusively in the cotton fiber.

Successive 5' deletions of the FSltp4 promoter linked to a GUS reporter gene were bombarded into a variety of cotton tissues (leaf, petal, epicalyx and 3–5 DPA fibers), which were then stained to detect GUS activity (Fig. 2, Table 1). The full-length FSltp4 promoter and the deletions down to –739 directed GUS expression only in the cotton fiber, demonstrating that the promoter region from –739 to –1,339 is not necessary for fiber specificity. The –624 deletion directed blue staining in both cotton fibers and petals, indicating that the promoter region from –624 to –739 is essential for restricting gene expression to the fiber. Further deletion to –410 also gave blue staining in petals and fibers. However, deletion to –320 abolished GUS expression in all of the tissues tested, showing that the region from –320 to –410 contains elements essential for promoter function. These elements must be in addition to the CAAT and TATA boxes in the core promoter, since the latter were retained in the –320 deletion.

Additional constructs were generated to define the promoter regions necessary for fiber-specific expression and minimal promoter activity more accurately. The –655 deletion directed GUS expression in cotton fibers (Fig. 2C, D) and the epicalyx (Fig. 2E, F), but not petals. This localized the promoter elements necessary for expression exclusively in the cotton fiber.
approximately 2.9 cotton fibers fused to the GAL4 with a cDNA library consisting of cDNAs from 8 DPA epicalyx, showing that elements necessary for gene expression in the fiber, petal and leaf, but not the enabling activity in the epicalyx. The –369 deletion directed elements that inhibit promoter activity in the petal while 639 and –755 (Fig. 3). This region also appears to contain fiber-specific expression to an AT-rich 84 bp FSR between –639 and –755 (Fig. 3). A yeast one-hybrid screen was used to isolate cotton fiber proteins that interact with the FSR. A yeast strain containing three direct tandem repeats of the FSR regulat-
ment involved the use of whole 3–5 DPA ovules from a minimum of four flowers (i.e. approximately 3.6 × 10⁶ fibers).+, Bombed tissue positive for GUS expression. –, bombarded tissue negative for GUS expression.

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Cotton tissues were bombarded with reporter plasmids containing GUS regulated by successive deletions of the FSltp4 promoter. The bombarded tissues were allowed to recover for 24 h and then histochemically stained for GUS expression. Bombardments were repeated in at least three independent experiments and tissues were scored as positive only when GUS expression was observed in at least two independent bombardments. Each cotton fiber bombard-
ment involved the use of whole 3–5 DPA ovules from a minimum of four flowers (i.e. approximately 3.6 × 10⁶ fibers).+, Bombed tissue positive for GUS expression. –, bombarded tissue negative for GUS expression.

The FSR interacts with a novel AT-hook protein (GhAT1) expressed in cotton fiber

A yeast one-hybrid screen was used to isolate cotton fiber proteins that interact with the FSR. A yeast strain containing three direct tandem repeats of the FSR regulating the lacZ or HIS3 gene was generated and transformed with a cDNA library consisting of cDNAs from 8 DPA cotton fibers fused to the GAL4 activation domain. Approximately 2.9 × 10⁶ clones were screened in the assay, and 29 HIS3- and lacZ-positive clones were isolated after three rounds of selection. Plasmid rescue and cDNA sequencing identified three types of cDNAs encoding FSR-interacting cotton fiber proteins, one of which (designated GhAT1) is described in the present work. Yeast clones containing GhAT1–GAL4 demonstrated strong growth on 4.5 mM 3-aminotriazole (3-AT) and significant lacZ activation when compared with control strains (Fig. 4A). The GhAT1 cDNA sequence did not encode a complete open reading frame due to truncation of the cDNA at the 5' end. A full-length cDNA was therefore isolated by 5'-RACE (rapid amplification of 5' cDNA ends) and cloning.

The full-length GhAT1 cDNA encodes a novel 340 residue protein with two AT-hook DNA-binding motifs (Fig. 5; GenBank accession No. EF140832). AT-hook motifs are the characteristic feature of high mobility group A (HMGA) and HMGA-like proteins (Bustin and Reeves 1996). Plant HMGA proteins typically contain four AT-hook motifs and an N-terminal domain with homology to the globular domain of histone H1 (Klosterman and Hadwiger 2002). HMGA-like proteins have a variable number of AT-hook motifs and contain additional domains (Klosterman and Hadwiger 2002). AT-hook motifs bind AT-rich sequences containing five or more bases via the minor groove of DNA (Bewley et al. 1998), and the specificity of protein binding is regulated by the length and sequence of the linker regions between the AT-hooks (Grasser 2003). GhAT1 is an HMGA-like protein with an N-terminal domain containing two AT-hook motifs, the first of which overlaps with a putative nuclear localization signal (Fig. 5). The C-terminus of GhAT1 contains a 118 amino acid domain of unknown function found in other AT-hook proteins from bacteria and Arabidopsis, pea, rice and other plants (GenBank domain DUF296, entry pfam03479). Several HMGA-like proteins have been shown to regulate promoter activity by interacting with AT-rich regulatory sequences (Grasser 2003), suggesting that GhAT1 might also function as a DNA-binding transcription factor.

**GhAT1** is nuclear localized and is highly expressed in cotton ovules

The subcellular localization of GhAT1 was determined by fluorescence microscopy after fusion of GhAT1 to a tag encoding green fluorescent protein (GFP) and bombard-
ment into onion epidermal cells. The GFP-GhAT1 fusion protein demonstrated strong nuclear localization (Fig. 4B), consistent with its function as a DNA-binding transcription factor. Cells bombarded with a control construct encoding a GFP–GUS fusion showed fluorescence throughout the cell, confirming that GFP alone has no intrinsic subcellular localization (Fig. 4B).

The expression of GhAT1 in cotton was assessed by reverse transcription–PCR (RT–PCR) and quantified by real-time PCR (Fig. 6). The RNA used in this analysis was derived from a number of cotton tissues including 0 DPA ovules; 6, 12, 18 and 24 DPA fibers (separated from the ovules); and fully expanded leaf, stem (from seedling epicotyls), whole flower (at anthesis) and
Fig. 2 Activity of the *FSltp4* promoter in cotton and transgenic tobacco. In situ hybridization of 0 DPA ovules (A and B). (A) Hybridization with an antisense RNA probe directed against *FSltp4*. Dark staining indicates the presence of *FSltp4* transcript. (B) Hybridization with a sense *FSltp4* RNA probe (negative control). GUS expression in cotton fibers bombarded with the −655 bp *FSltp4::GUS* promoter deletion construct (C–F). (C) GUS staining in a cluster of cotton fibers (scale bar = 0.34 mm). (D) A single stained cotton fiber (scale bar = 0.34 mm). (E) Epicalyx showing spots of GUS expression designated by arrows (scale bar = 1.1 mm). (F) Detail of the boxed region in (E). GUS expression is indicated by an arrow; the remaining dark spots are oil glands. Scale bar = 0.42 mm. GUS expression in mature *FSltp4::GUS* transgenic tobacco (G–L). (G) Tobacco leaf showing GUS expression in trichomes, vascular tissues and leaf margin (scale bar = 2.1 mm). (H) GUS staining in glandular trichomes on the abaxial surface of the leaf midvein. Also note the staining in midvein vascular tissue (scale bar = 0.53 mm). (I) GUS expression in simple and branched trichomes (scale bar = 2.1 mm). (J) GUS staining in anther and anther trichomes (examples shown by arrows). Scale bar = 0.71 mm. (K) GUS staining in the stigma, anthers and petal margins (scale bar = 1.1 mm). (L) GUS activity in ovules and capsule (scale bar = 0.71 mm). GUS expression in 21-day old T₂ seedlings (M–O). (M) GUS expression in a root tip and lateral root primordium (scale bar = 0.34 mm). (N) The leaf margins, vascular tissue and axillary shoot primordia demonstrate strong GUS expression (scale bar = 2.1 mm). (O) Detail of GUS expression in axillary shoot primordia (arrowed) (scale bar = 0.34 mm).
root (from seedlings). *GhAT1* expression was highest in the 0 DPA ovules. Whole flower, leaf and stem demonstrated moderate levels of *GhAT1* expression, while minimal expression was observed in root and cotton fiber. This result suggests that *GhAT1* may regulate the *FSlt4* promoter in ovules and other non-fiber tissues.

**GhAT1 represses the activity of the FSltp4 promoter in tobacco leaf trichomes**

The transcriptional function of GhAT1 was analyzed in transgenic tobacco. An *FSlt4::GUS* tobacco line was transformed with a 35S::*GhAT1* construct to generate *FSlt4::GUS* plants with constitutive expression of *GhAT1*. PCR analysis of transformants confirmed two independent lines of *FSlt4::GUS/35S::GhAT1* tobacco (Fig. 7A). Quantitative analysis of GUS activity in whole leaves revealed substantial (15-fold) repression of the *FSlt4* promoter in both lines (Fig. 7B). Histochemical staining of *FSlt4::GUS/35S::GhAT1* leaves demonstrated that GUS expression was absent from most trichomes and from the leaf margin, but was similar to *FSlt4::GUS* tobacco in the vascular tissue (Fig. 8A–C). A few stained trichomes were also observed across the leaf surface (Fig. 8D). These results demonstrate a specific interaction between the *FSlt4* promoter and GhAT1 in planta and show that GhAT1 is a negative regulator of the trichome-specific activity of the promoter.

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**Fig. 3** Analysis of *FSlt4* promoter activity. (A) Structure of *FSlt4::GUS* reporter constructs. The *FSlt4* promoter (or a 5′ deletion of the promoter) was cloned into the SacI and *Bam*HI sites of pJKKmf(−):GUS.ocs (Kirschman and Cramer 1988) to place GUS under the control of the promoter sequence. (B) Schematic diagram showing *FSlt4* promoter sequences cloned into pJKKmf(−):GUS.ocs. The fiber specificity and activation regions of the promoter (as identified using transient expression in cotton tissues) are shaded. The transcription start site (+1) corresponds to that shown in (A).

**Fig. 4** Isolation and subcellular localization of GhAT1. (A) Isolation of GhAT1 cDNA in a yeast one-hybrid assay. Target-reporter constructs containing three copies of the FSR regulating the expression of *HIS* or *lacZ* were integrated into the YM4271 yeast genome to generate strain FSR3x. A cDNA library from 8 DPA cotton fiber was ligated into a yeast expression vector to generate a library of plasmids encoding fiber cDNA–GAL4 activation domain fusion proteins. This library was transformed into FSR3x. Clones encoding putative DNA-binding cDNAs were then isolated by their *lacZ* activity and their ability to grow on selective synthetic drop-out (SD) medium (SD –Leu/–His + 4.5 mM 3-AT). False-positive clones, such as the 40S ribosomal protein (shown as Ribosomal/FSR3x) and FSR3x, were incapable of growth on SD –Leu/–His + 4.5 mM 3-AT. The clone encoding partial GhAT1 demonstrated strong *lacZ* expression in filter lift assays using X-Gal (visible as blue staining), while FSR3x showed no background *lacZ* expression. (B) Plasmids containing genes encoding either a GFP–GhAT1 or GFP–GUS fusion protein were introduced into onion cells by particle bombardment, and fluorescent cells were imaged by epifluorescence. The scale bar is 135 μm for cells bombarded with GFP–GhAT1 plasmid and 160 μm for cells bombarded with GFP–GUS control plasmid.
Fiber-specific promoters have a major role in the regulation of cotton fiber development and are important tools in the genetic manipulation of fiber quality. Promoter analysis in stably transformed transgenic cotton is difficult, and this has encouraged the use of transgenic Arabidopsis and tobacco as model systems for the analysis of fiber-specific promoters. The FSltp4 promoter demonstrated strong fiber-specific activity in cotton, but was active in both trichome and non-trichome tissues in transgenic tobacco in a pattern consistent with LTP function. These results suggest that fiber and trichome specificity are regulated by distinct mechanisms in cotton and tobacco. Hence, while tobacco and Arabidopsis are useful model systems for promoter analysis, cotton provides a more direct approach to understanding the regulation of fiber-specific genes.

**Discussion**

Fiber-specific promoters have a major role in the regulation of cotton fiber development and are important tools in the genetic manipulation of fiber quality. Promoter analysis in stably transformed transgenic tobacco is difficult, and this has encouraged the use of transgenic Arabidopsis and tobacco as model systems for the analysis of fiber-specific promoters. The FSltp4 promoter demonstrated strong fiber-specific activity in cotton, but was active in both trichome and non-trichome tissues in transgenic tobacco in a pattern consistent with LTP function. These results suggest that fiber and trichome specificity are regulated by distinct mechanisms in cotton and tobacco. Hence, while tobacco and Arabidopsis are useful model systems for promoter analysis, cotton provides a more direct approach to understanding the regulation of fiber-specific genes.
systems for the analysis of fiber specificity, results in these systems may not be directly applicable to cotton.

Transient transformation of cotton tissues by microprojectile bombardment was used to avoid this problem and the limitations associated with promoter analysis in transgenic cotton. The transient assay demonstrated that an 84 bp FSR is necessary for confining gene expression exclusively to the cotton fiber, while a 49 bp AR (and a 369 bp minimal promoter) is necessary for promoter activity in all of the tissues tested. Liu et al. (2000) demonstrated that a 315 bp region of the Ltp3 promoter is necessary for trichome-specific promoter activity in transgenic tobacco.

Fig. 7 Analysis of FSltp4::GUS/35S::GhAT1 tobacco. (A) PCR analysis. The transgenic status of each FSltp4::GUS/35S::GhAT1 line was confirmed by amplification of sequences from the FSltp4 promoter and GhAT1 (739 and 704 bp, respectively). The template was genomic DNA from wild-type (WT), FSltp4::GUS/35S::GhAT1 line 1 (1), FSltp4::GUS/35S::GhAT1 line 2 (2) or FSltp4::GUS (3) tobacco. The negative control reaction (−) contained no template. Plasmids containing FSltp4::GUS or GhAT1 (as appropriate) were used as template in the positive control reactions (+). (B) Quantitative measurement of GUS activity. Total protein was extracted from size-matched (22 cm) leaves of FSltp4::GUS and FSltp4::GUS/35S::GhAT1 tobacco (lines 1 and 2). GUS activity was measured according to Rao and Flynn (1992) and is shown as picomoles of product (4-methylumbelliferone; 4-MU) generated per hour per µg of total leaf protein extract. Values shown are the average activity of three samples of protein extract. Error bars show the standard deviation.

Fig. 8 GUS expression in FSltp4::GUS/35S::GhAT1 tobacco. Size-matched (17 cm) leaves were histochemically stained for GUS activity. (A) GUS expression in trichomes, leaf margin and vascular tissue of FSltp4::GUS control. (B–D) GUS expression in FSltp4::GUS/35S::GhAT1 leaves. Most trichomes are negative for GUS expression (B and C), although a small number of trichomes show staining (D). The leaf margin is negative for GUS activity, but expression is retained in the vascular tissue (B and C). Scale bar = 1.4 mm (A, B), 2.1 mm (C) or 0.44 mm (D).
This region is located at −614 to −299, and aligns with 311 bp of the FSltp4 promoter at −608 to −297 (Fig. 1A). However, the 311 bp FSltp4 sequence does not include the 84 bp FSR, which is located at positions −755 to −639. Given that the Lip3 and FSltp4 promoters show substantial nucleotide similarity, this result suggests that fiber- and trichome-specific gene expression is regulated by distinct promoter elements.

The FSR is AT-rich (77% AT), and AT-rich sequences have been shown to regulate the tissue specificity of a number of plant promoters (e.g. Czarnecka et al. 1992, Webster et al. 1997, Terce-Laforgue et al. 1999). However, analysis of other fiber-specific promoters (both LTP and non-LTP) with PLACE (Higo et al. 1999) and other motif prediction programs failed to identify promoter sequences similar to the FSR or any common pattern of potential regulatory sequences (Harmer et al. 2002, S. Orford unpublished results). This may be attributable to the limited availability of biochemical and genetic data on plant (particularly cotton) transcription factors, and the consequently low power of promoter element prediction and comparison programs (Tompa et al. 2005). Nevertheless, gene expression studies provide strong evidence for the coordinate regulation of subsets of genes at specific stages in fiber development (Smart et al. 1998, Arpat et al. 2004), and indicate that fiber-specific promoters are likely to be controlled by common transcriptional mechanisms.

Deletion of the FSR did not prevent gene expression in the fiber, but rather extended gene expression to other tissues. This suggests that fiber-specific gene expression is regulated by at least two sets of transcription factors: those that restrict gene expression to the cotton fiber, and those that are essential for promoter activity in both the fiber and other cell types. The 49 bp AR does not contain any known cis-acting promoter elements, but is likely to bind those transcription factors that are essential for gene expression in the fiber.

The activity of the FSltp4 promoter in fiber initials (Fig. 2A, B) suggests that FSltp4 may be involved in fiber initiation in addition to its apparent function in fiber elongation (Orford and Timmis 1997). GhAT1 is transcribed at a high level in 0 DPA ovules and the mRNA is present in many non-fiber tissues, but transcripts are rare in elongating fibers. The GhAT1 protein negatively regulates the FSltp4 promoter in trichomes, and so GhAT1 may regulate cotton fiber development by repressing fiber-specific genes in non-fiber tissues. This repression may be particularly important in the determination of cell fate during fiber initiation, since GhAT1 is expressed at a higher level in 0 DPA ovules than in other non-fiber tissues. This conclusion also suggests the possibility that repression of GhAT1 in the ovule epidermis could increase fiber initiation by activating the expression of fiber-specific genes in the epidermal cells.

GhAT1 is functionally and structurally distinct from the MYB and HOX transcriptional activators that have been implicated previously in fiber and trichome specificity and initiation (Pesch and Hulskamp 2004, Wang et al. 2004). HMGA-like proteins similar to GhAT1 are involved in the transcriptional regulation of a number of plant promoters (Tjaden and Coruzzi 1994, Meijer et al. 1996, Reisdorf-Cren et al. 2002, Matsushita et al. 2007), suggesting that they may have an important general role in the regulation of plant gene transcription. HMGA proteins have been shown to regulate promoter activity by interacting with other proteins and/or by inducing changes in DNA conformation (Martinez-Garcia and Quail 1999, Grasser 2003), and HMGA-like proteins may influence promoter activity using similar mechanisms (Reisdorf-Cren et al. 2002). Further analysis of GhAT1 and FSR function would provide greater insight into the transcriptional regulation of fiber development and an improved capacity to modify gene expression in the cotton fiber.

Materials and Methods

Isolation of FSltp4 promoter

The 5′-flanking region of FSltp4 was isolated from cotton (Gossypium hirsutum cv. Siokra 1–4) genomic DNA using the Universal GenomeWalker PCR kit (Clontech, Carlsbad, CA, USA). Nested primers were designed from the pFS6 cDNA sequence and applied to total genomic DNA according to the manufacturer's protocol. Southern analysis was then performed on the PCR products with a pFS6 cDNA probe, and hybridizing fragments of the appropriate size were cloned into pGEM-TEasy (Promega, Madison, WI, USA). The initial primers were P6PRA (5′-GGCTTCCACTAGCTGGTGAACCTTCC-3′) and P6PRB (5′-CGGCATAGTACACAACCCACAACTCTGG-3′). Follow-

Preparation of FSltp4 promoter deletions

The FSltp4 promoter sequence was analyzed against the PLACE database (Higo et al. 1999) for potential transcription factor-binding sites. A series of 5′ promoter deletions flanking these sites was then generated by PCR using the antisense primer p61 (5′-GCGGTTCCACTAGCTGGTGAACCTTCC-3′) and the following sense primers: p60, 5′-CCGAAGCTTACTGCTCTTGACAT TGC-3′; p62, 5′-CCGAAGCTTACTGCTCTTGACAT TGC-3′; p63, 5′-CCGAAGCTTAATGGAATATGCAGT CTG-3′; p64, 5′-CCGAAGCTTAATGGAATATGCAGT CTG-3′; p65, 5′-CCGAAGCTTACATGGAATATGCAGT CTG-3′; p66, 5′-CCGAAGCTTACATGGAATATGCAGT CTG-3′.

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PCR cycling conditions were 2 min at 95°C followed by 25 cycles of 1 min at 95°C, 1 min at 55°C and 4 min at 72°C. Deletions containing terminal HindIII and BamHI sites were cloned into pGEM-Teasy (Promega), excised with SacI and BamHI, and subcloned into the promoter-less GUS expression vector pJKKmf(-)-GUS.ocs (Kirschman and Cramer 1988), while deletions with terminal SacI and BamHI sites were cloned directly into the expression vector. This generated a series of constructs containing a GUS expression cassette regulated by an FStlp4 promoter deletion (Fig. 3). Deletions were numbered in the 5’ direction from the first nucleotide in the FStlp4 start codon (defined as +1). The full-length FStlp4::GUS reporter construct was designated as pJK-1339, and the deletions were pJK-960, –865, –739, –655, –624, –511, –410, –369 and –320.

Plant materials and bacterial strains

Cotton plants (G. hirsutum cv. Siokra 1-4 and cv. Coker 315) were maintained in growth cabinets at temperatures of 30°C (day) and 25°C (night) with a day/night cycle of 16/8 h. Plants were illuminated by mercury vapor growth lamps and were grown in soil at a density of 2–4 per 8 liter pot. Tobacco plants (N. tabacum cv. Wisconsin 38) were grown under similar conditions at temperatures of 22°C (day) and 20°C (night) with a day/night cycle of 16/8 h. Tobacco tissue cultures were grown on Murashige-Skoog (MS) medium at a constant temperature of 25°C under fluorescent lights with a day/night cycle of 16/8 h. Onion (Allium cepa) samples of each other tissue. At least two independent bombardments were integrated into the genome of a single yeast strain (designated FSR3x). The yeast one-hybrid assay was then performed by transforming FSR3x with a cDNA library derived from 5 DPA cotton fibers. Library construction and transformation were performed using the Yeastmaker Yeast Transformation System 2 (Clontech) and the MatchMaker cDNA Library Construction Kit (Clontech) in accordance with the manufacturer’s instructions.

RT-PCR and real-time RT-PCR

cDNA samples for use in RT-PCR and real-time RT-PCR were obtained by performing reverse transcription on mRNA isolated from a number of cotton tissues. These were 6, 12, 18 and 24 DPA cotton fibers (separated from the ovules); 0 DPA ovule; whole flower (collected at anthesis); and stem (epicotyl) and root from seedlings. Briefly, total RNA was isolated from cotton tissues by a modified hot borate method (Wan and Wilkins 1994) and reverse transcription was performed as previously described (Harmer et al. 2002). Parallel reactions with and without reverse transcriptase were generated for each DNase-treated RNA sample. The –RT samples failed to produce any product in either RT–PCR or real-time RT–PCR, demonstrating that amplification in the +RT samples was not attributable to DNA contamination (data not shown). Note that cDNA samples derived from the same tissues were used for both RT–PCR and real-time RT–PCR.

RT–PCRs were performed as described (Harmer et al. 2002) with the following cycling parameters: denaturation for 2 min at 95°C; 28 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C and extension at 72°C for 2 min; and a final cycle with an extension time of 4 min (total 29 cycles). G3AT1 was amplified with the primers 5’-AATCGGTTTAAAAATGG ATCGAAGGGATGC-3’ and 5’-CTTATGTTGCCCTGGACTATGAGC-3’. Actin was amplified as an internal standard according to Shimizu et al. (1997) using the primers 5’-AATCGGTTTAAAAATGG ATCGAAGGGATGC-3’ and 5’-CTTATGTTGCCCTGGACTATGAGC-3’. Real-time PCR was performed in 25 μl reactions containing 0.5 SYBR Green PCR Master Mix (Applied Biosystems,
Warrington, UK), 0.4 μM of each primer and 0.5 μl of first-strand cDNA (corresponding to 50 ng of total RNA). Reactions were run using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and associated software with the following cycling parameters: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Dissociation curves (from 60 to 95°C) were calculated after cycling using the manufacturer’s default dissociation protocol. Three replicates were performed for each cDNA–primer combination in each run. *GhAT1* was amplified using the primers AT11F (5′-TGACATTTTGCACGCAATGGA-3′) and AT11R (5′-CGGCTTTCGAA GAGTTACGG-3′). The *GhACT3* actin gene is expressed at approximately equal levels in different cotton tissues (Li et al. 2005), and was used as internal standard for relative quantification. *GhACT3* was amplified using the primers GhACT3F (5′-GGCCCTGAGCAATGGGAC-3′) and GhACT3R (5′-AACAGATGAGCTGCTCTTG-3′). The *GhAT1* and *GhACT3* amplifications were performed in each run to minimize technical variation.

5′-RACE

The *GhAT1* cDNA fragment isolated in the yeast one-hybrid assay did not encode a full-length protein and so 5′-RACE was used to isolate the complete cDNA. A cDNA library was constructed from 12 DPA cotton fiber using the Marathon cDNA Amplification Kit (Clontech), and 5′-RACE was performed according to the manufacturer’s protocol using the adaptor primer API (5′-CCATCTAATAGCTAAGATAGGCC-3′) and the gene-specific primer ATR1 (5′-GATTGCGAGAGCCAGTA TGCTTTGCTC-3′). An 847 bp PCR product was isolated and cDNA–primer combination in each run. *GhAT1* was sequenced to demonstrate that the fragment contained 402 bp of sequence identical to the initial *GhAT1* fragment and 472 bp of additional 5′ sequence. The complete *GhAT1* cDNA was obtained by ligating together the cDNA fragments from the yeast one-hybrid assay and 5′-RACE using an *SnaBI* site located in the region of overlap between the two sequences. An 899 bp *SnaBI*–*SacI* fragment containing the 3′ end of *GhAT1* was excised from the yeast one-hybrid vector pGADT7-Rec and cloned into pAT11. This produced a plasmid containing the full-length *GhAT1* cDNA (pAT1801).

Construction and analysis of GFP fusion proteins

The *GhAT1* cDNA was amplified from pAT18101 by PCR using the primers AT11F (5′-AATCGGTTTAAAATGGATCGA) and AT11R (5′-CGTGAATTCTTACGCATAT) (Clontech). The resultant PCR product was digested with *DraI* and *EcoRI* and cloned into the Gateway entry vector pENTR1A (Invitrogen, Carlsbad, CA, USA). The *GhAT1* cDNA fragment was then sequenced to ensure nucleotide identity and transferred to the destination vector pMDC43 (Curtis and Grossniklaus 2003) using the Gateway LR recombination system (Invitrogen). The destination vector was linearized with *KpnI* prior to LR recombination to increase reaction efficiency. Recombination was performed in accordance with the manufacturer’s instructions, although the reaction was incubated for approximately 30 h at room temperature. Following recombination, the reaction was cleaned up using a gel extraction kit (Qiagen, Valencia, CA, USA), self-ligated to re-circularize the destination vector and digested with *ApaI* to remove the entry vector. The resultant reaction was transformed into *Escherichia coli* and plasmids were recovered and analyzed for the presence of the *GhAT1* cDNA by restriction analysis with *DraI* and *EcoRI*. This procedure resulted in a plasmid (pMDC43GhAT1) containing the *GhAT1* cDNA fused in-frame to the N-terminus of GFP.

A positive control plasmid (pMDC43GUS) encoding a GUS–GFP fusion was generated by transferring GUS from pENTR-gus to pMDC43 by LR recombination. The pMDC43GUS and pMDC43GhAT1 plasmids were bombarded into onion cells and GFP expression was visualized by fluorescence microscopy.

Quantitative measurement of GUS activity

The GUS activity of total protein extracts from tobacco leaves was measured essentially as described (Rao and Flynn, 1992) using 4-methylumbelliferyl-β-D-glucuronide (Sigma-Aldrich, St Louis, MO, USA) as the GUS substrate.

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References


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