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A R2R3 MYB Transcription Factor GhMYB109 is Required for Cotton Fiber Development

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ABSTRACT

Cotton (*Gossypium hirsutum* L.) fibers are single highly elongated cells derived from the outer epidermis of ovules. A large number of genes are required for fiber differentiation and development, but so far, little is known about how these genes control and regulate the process of fiber development. Here we examine the role of a cotton fiber-specific R2R3 MYB gene *GhMYB109* in cotton fiber development. Transgenic reporter gene analysis revealed that a 2-kb *GhMYB109* promoter was sufficient to confirm its fiber-specific expression. Antisense-mediated suppression of *GhMYB109* led to a substantial reduction in fiber length. Consistently, several genes related to cotton fiber growth were found to be significantly reduced in the transgenic cotton. Our results showed that the *GhMYB109* is required for cotton fiber development and reveal a largely conserved mechanism of R2R3 MYB transcription factor in cell fate determination in plants.

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is an important economic crop that is extensively used in the textile industry. Cotton fibers are single-celled trichomes derived from epidermal cells of the ovule (BASRA and MALIK 1984). The fiber development usually consists of four overlapping stages: initiation, primary cell wall formation, secondary cell wall formation and maturation. During the initial stage, approximately 30% of epidermal cells (fiber initials) on the ovule surface begin to enlarge and elongate rapidly at or just before anthesis. The primary cell wall formation starts at anthesis and lasts up to 19-20 days postanthesis (DPA) (BASRA and MALIK 1984). The quality and productivity of cotton fibers depend mainly on two biological processes: fiber initiation to determine the number of fibers present on each ovule and fiber elongation to control the final length and strength of each fiber (JOHN and KELLER 1996). Synthesis of the secondary wall initiates about 16 DPA, overlapping with the late primary wall formation, and continues for about 40 DPA, forming a wall (5-10 μm thickness) of almost pure cellulose. Upon maturity, cotton fibers contain about 90% cellulose. Thus, research of fiber development not only provides the basic understanding of cell differentiation and elongation, but also identifies potential target genes for genetic improvement of cotton fiber production.

Cotton fibers are seed trichomes, which share many similarities with leaf trichomes. Based on *Arabidopsis thaliana* trichome and cotton fiber both are single-celled hairs of epidermal origin, it is likely that Arabidopsis trichomes could

serve as a model for elucidating the genetic mechanisms controlling cotton fiber development (SERNA and MARTIN 2006). For the model plant Arabidopsis, trichome development and root epidermal patterning have been studied in depth, and both processes use a common mechanism involving closely related transcription factors and a similar lateral inhibition signaling pathway (LARKIN *et al.* 2003; SCHNEIDER *et al.* 1997; SCHNITTGER *et al.* 1999). Transcription factors such as MYB proteins GLABRA1(GL1) or WEREWOLF(WER), WD40 proteins TRANSPARENT TESTA GLABRA1 (TTG1), and basic helix-loop-helix proteins GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3) appear to form a transcription factor complex to determine epidermal trichome patterning in Arabidopsis (GLOVER 2000; HULSKAMP 2004; RAMSAY and GLOVER 2005; SCHIEFELBEIN 2003; SERNA and MARTIN 2006). This complex is thought to regulate a homeodomain leucine zipper protein GLABRA2 (GL2) and a small family of single-repeat MYB proteins lacking transcription activation domains TRIPTYCHON (TRY), CAPRICE (CPC) and ENHANCER OF TRY AND CPC1 (ETC1). *GL2* encodes a homeobox (HOX) transcription factor that promotes trichome cell differentiation and growth (OHASHI *et al.* 2002; RERIE *et al.* 1994; SZYMANSKI *et al.* 1998). Single-repeat MYB proteins TRY, ETC1and ETC2 have been shown to negatively regulate trichome formation and act in a partially redundant manner to mediate the lateral inhibition (KIRIK *et al.* 2004a; KIRIK *et al.* 2004b; SCHELLMANN *et al.* 2002; SCHNITTGER *et al.* 1999). Similar genes and pathways may be involved during seed trichome development in cotton, although cotton fibers are unicellular and never branch.

Compared with the Arabidopsis trichome, little is known about the molecular control of the cotton fiber development. Recent studies on cotton fiber development have been focused largely on gene expression profiles during fiber elongation and secondary cell wall synthesis (ARPAT *et al.* 2004; SHI *et al.* 2006; TALIERCIO and BOYKIN 2007; UDALL *et al.* 2006; WU *et al.* 2006; YANG *et al.* 2006). Previous results suggested that transcription factors could play important roles in cotton fiber development. So far, a dozen of genes encoding transcription factors are found to be expressed in developing cotton fiber cell, and some of them show similarity to Arabidopsis trichome regulators in protein sequences. An earlier work isolated 6 MYB genes (*GhMYB1-GhMYB6*) from *G. hirsutum* (LOGUERICO *et al.* 1999). Another cotton R2R3 MYB gene *GaMYB2* complements the Arabidopsis *gl1*, and its ectopic expression induces a single trichome from the epidermis of Arabidopsis seeds (WANG *et al.* 2004b). *GhMYB25,* a homolog of *AmMIXTA/AmMYBML1* that controls petal conical cell and trichome differentiation in *Antirrhinum majus*, is predominately expressed in ovules and fiber cell initials (WU *et al.* 2006). A recent work has shown that a gene similar to *AtCPC* that acts as an inhibitor of trichome development in Arabidopsis was identified in fiber initials and appeared to possess the MYB domain but lack the transacting domain similar to its Arabidopsis counterpart (TALIERCIO and BOYKIN 2007). Four putative homologues of *TTG1*, *GhTTG1-GhTTG4* from *G. hirsutum* are found to be widely expressed in plant tissues, including ovules and fibers. Two of them were able to complement the Arabidopsis *ttg1* mutant (HUMPHRIES *et al.* 2005). Nevertheless, the exact function of these genes in cotton fiber development is

not clear. Obviously, cotton fiber cell development is a complex biological process that requires orchestrated changes in gene expression in developmental and physiological pathways (ARPAT *et al.* 2004; JI *et al.* 2003; KIM and TRIPLETT 2001; LEE *et al.* 2006; LI *et al.* 2002).

Many cotton genes with a fiber-preferential expression have been cloned and characterized. For example, *GhTUB1* gene was preferentially expressed in the elongation stage of fiber development (LI *et al.* 2002). Fifteen *GhACT* cDNAs were found to be differentially expressed in various tissues. Specifically, *GhACT1* has been found to be predominantly expressed in fiber cells, and its suppression disrupted the actin cytoskeleton and caused reduced fiber elongation, suggesting that *GhACT1* plays an important role in fiber elongation but not fiber initiation (LI *et al.* 2005). A recent study revealed that the *1-Aminocyclopropane-1-Carboxylic Acid Oxidase1-3* (*GhACO1-GhACO3*) gene, which is responsible for ethylene production, is expressed at a significantly higher level in rapidly elongating fiber cells, indicating a role of ethylene in cotton fiber cell elongation (SHI *et al.* 2006). Although several of these genes are involved in fiber development, none of them encode a transcription factor regulating fiber development.

So far, the molecular control of cotton fiber development remains largely unknown, though the cotton is the most important fiber crop for the textile industry. Current understanding of cotton fiber development is limited to computational and expression analyses of high-quality ESTs, and the isolation and characterization of fiber-related genes. Therefore, deciphering the molecular control of fiber development

will be important for cotton improvement by genetic engineering. In this study, we examined the role of *GhMYB109* (SUO *et al.* 2003), similar to *AtGL1/WER*, in cotton fiber development using a reverse genetics approach. Our results provide an insight into the molecular mechanism regulating cotton fiber development and reveal a largely conserved mechanism in cell fate determination in plants.

MATERIALS AND METHODS

Plant materials and growth conditions: Cotton (*Gossypium hirsutum* cv Coker312 and *G. hirsutum* L. cv. XZ142) seeds were surface-sterilized with 70% ethanol for 30 to 60 sec and 10% H_2O_2 for 30 to 60 min, followed by washing with sterile water. Sterilized seeds were germinated on half-strength MS medium under a 16-h-light/8-h-dark cycle at 28°. Cotyledons and hypocotyls were cut from sterile seedlings as explants for transformation. Tissues for DNA and RNA extraction were derived from cotton plants grown in a greenhouse. Vegetative and reproductive organs and tissues were harvested from the cotton specie *G. hirsutum* L. cv. XZ142 grown under a 30/21° day/night temperature regime in greenhouse. Developing ovules were excised from developing flower buds or bolls on various days before/post-anthesis (DPA) relative to the day of anthesis (0 DPA).

Genome Walker PCR and GUS reporter construct: The unknown regions of the 5' putative promoter and 3' end of *GhMYB109* were determined using the Universal Genome Walker kit (Clontech, Palo Alto, Calif.). Briefly, genomic DNA of *G. hirsutum* L. cv. XZ142 was digested with *Eco*RV, *Dra*I, *Pvu*II, *Stu*I, and *Sca*I,

respectively. DNA fragments were ligated with a Genome Walker adaptor (5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3' and 3'-H_2N-CCCGACCA-PO_4-5'), which had one blunt end and one end with a 5' overhang. The primary PCR was performed using an adaptor primer AP1 (5'-GTAATACGACTCACTATAGGGC-3', forward) and *GhMYB109*-specific primers GW1 (5'-GAAGTGTGACTGTGTTGTTAAGAACCTG-3', reverse) for the *GhMYB109* promoter. The secondary PCR was performed using primer AP2 (5'-ACTATAGGGCACGCGTGGT-3', forward) and a nested gene-specific primer GW2 (5'-GAGTAACTTGTCTTCCTCCATTGCCCATAAT-3', reverse). The 3' end of *GhMYB109* was analyzed in a similar way using primer AP1 and GW3 (5'-GACCATGATTATGAGCTAAGTACACTTGCC-3', reverse) for primary PCR and AP2 and GW4 (5'-GTACACTTGCCATGATTGACCACTTCCATG-3', reverse) for secondary PCR. Then 2-kb putative promoter of *GhMYB109* was amplified using two primers (5'-ATAGTCGACTGTGTCAAAGACGACTACTTGAG-3', forward) and (5'-TCTAGAGAGTAACTTGTCTTCCTCCATTGCCCATAAT-3', reverse).The 2-kb 3'-terminator sequences of *GhMYB109* was obtained using two primers (5'-ATGAATTCTATGCTGAGCTTGCCAAGGG-3', forward) and (5'-ATGAGCTCCATCTTAGCTAGAGACTATGTTAT-3', reverse). The putative promoter region was inserted upstream and the 3'-terminator was inserted downstream of the β-*glucuronidase* (*GUS*) reporter gene in *pBI101.2* vector (Clontech, CA, USA), giving rise to the *GhMYB109::GUS* fuse gene. The construct was completely sequenced to ensure that it did not contain any PCR or cloning errors and used for

cotton transformation.

Plasmid constructs: The coding region of *GhMYB109* was subcloned into appropriately digested *pBI121* vector (Clontech, CA, USA) in the antisense orientation, downstream of the cauliflower mosaic virus (CaMV) 35S promoter. The primers used were as follows: 5'-ATAGAGCTCATGGCCGGGGATACAAAAAGG-3' (forward) and 5'-TATTCTAGACCCGAATCTAATAACATAGTC-3' (reverse). The constructs were completely sequenced to ensure that they did not contain any PCR or cloning errors and used for cotton transformation.

Cotton transformation: Cotton transformation was performed as previously described (LI *et al.* 2005). The constructs were introduced into *Agrobacterium* strain AGL-1 used for transformation. Cotyledon and hypocotyl explants from *G. hirsutum* cv Coker 312 were transformed using *Agrobacterium*–mediated transformation. Homozygosity of transgenic plants was determined by segregation ratio of kanamycin selection marker and further confirmed by DNA gel blot, real-time PCR, RT-PCR, and histochemical assay.

Histochemical assay of GUS gene expression: Histochemical assays for GUS activity in transgenic cotton plants were conducted as described previously (WANG *et al.* 2004a). The samples were cut into 5-to 7-mm-thick sections using a Leica microtome. The sections were examined and photographed under a Leica DMR microscope equipped with dark-field optics.

Scanning electron microscopy: For examining fiber initiation and elongation,

fresh ovules were dissected out and placed on double-sided sticky tape on an aluminum specimen holder and frozen immediately in liquid nitrogen. The frozen sample was viewed with a JSM-5310LV scanning electron microscope (JEOL, Tokyo, Japan). Fiber density in the stage of initiation was estimated by counting fiber initials per unit area of 100μm×100μm using a total of 25 unit areas per ovule from the epidermis of ovule under SEM and statistically analyzed. Eight or nine ovules were used for the transgenic and wild-type plants.

DNA gel blot analysis: Cotton genomic DNA isolation and Southern blotting analysis were performed as described previously (SUO *et al.* 2003). Genomic DNA (20μg) was digested, separated on 0.8% agarose gel and transferred onto Hybond N+ membrane (Amersham, Buckinghamshire, UK). DNA gel blot analysis of *G. hirsutum* cv Coker 312 and transgenic cottons were carried out using *NPTII* and *GhMYB109* cDNA as probes.

Real-time PCR: The expression of the *GhMYB* genes and other fiber-related genes in cotton tissues was analyzed by real-time quantitative RT-PCR (qRT-PCR). From a pool of 3-4 plants of each line, the bolls were tagged and harvested at the day of anthesis (0 DPA), 1 day post-anthesis (1 DPA), and 3 DPA. Total RNA was extracted from immature ovules or fiber-bearing ovules as previously described and digested with DNase I (TaKaRa, Dalian, China) (SUO *et al.* 2003). qRT-PCR was performed as previously described in all experiments (LAN *et al.* 2004). In brief, two micrograms of total RNA was used for cDNA synthesis with SuperScript III First-Strand Synthesis kit (Invitrogen, CA, USA). The cDNA samples were diluted to

8 and 2ng/µl. Triplicate quantitative assays were performed on 1 µl of each cDNA dilution using the SYBR Green Master Mix (Applied Biosystems, CA, USA) with an ABI 7900 sequence detection system according to the manufacture's protocol (Applied Biosystems). Gene-specific primers (Table 1) were designed by using PRIMEREXPRESS software (Applied Biosystems). The relative quantification method (DDCT) was used to evaluate quantitative variation between replicates examined using p-value≤0.05 and fold change of expression levels ≥2-fold change as cut-off. Amplification of 18S rRNA was used as an internal control to normalize all data.

RESULTS

The *GhMYB109* promoter is cotton fiber-specific: Our previous study showed that a R2R3 MYB transcription factor GhMYB109 was found to be structurally related to AtGL1 and AtWER controlling the trichome initiation in *A. thaliana*. Our previous study also found that *GhMYB109* was specifically expressed in cotton fiber initial cells as well as elongating fibers (SUO *et al.* 2003). To better define the expression pattern of *GhMYB109* in cotton fibers, a 2-kb putative promoter and a 2-kb 3'-terminator sequences of *GhMYB109* were inserted downstream of the β-*glucuronidase* (*GUS*) reporter gene in *pBI101.2* vector, giving rise to the *GhMYB109*::*GUS* fusion gene (Figure 1A). The *GhMYB109*::*GUS* construct was introduced into the genome of cotton cultivar Coker312 by *Agrobacterium tumefaciens*-mediated transformation. Twenty progeny from five independent

transgenic lines were examined in detail for the *GUS* expression pattern, using nontransformed wild-type plants as a negative control. In each line, a strong GUS activity was observed only in fibers (Figure 1, B, C, E, F and G), whereas no or little GUS staining was detected in ovules, petals, sepals, leaves, stems and flower buds before anthesis (Figure 1H). In comparison, nontransformed plants showed no GUS activity in fibers (Figure 1D) nor in other tissues under the same staining regimen (data not shown). The same pattern of the *GhMYB109::GUS* expression was also found in T_1 and T_2 transgenic plants (data not shown). These results indicated that the 2-kb *GhMYB109* putative promoter was sufficient to direct the fiber specific expression of the *GUS* reporter gene, confirming that it is a fiber-specific gene.

Generation of antisense *GhMYB109* transgenic plants: To examine the role of *GhMYB109* in fiber development, an antisense *GhMYB109* transformation vector driven by the cauliflower mosaic virus (CaMV) 35S promoter (Figure 2A) was constructed and introduced into cotton cultivar Coker312 by *Agrobacterium tumefaciens*-mediated transformation. Two independent transgenic T_1 lines were subsequently obtained. DNA gel blot analysis using *NPTII* and *GhMYB109* cDNA as probes confirmed that lines AS24-1 and AS24-2 (same transformation event) had two copies and the other line AS54-1 one copy of the antisense *GhMYB109* (*35S::GhMYB109AS*) transgene (Figure 2, B and C), consistent with the sites of enzymes in genomic DNA and construct.

To examine the expression of *GhMYB109* in the two *35S::GhMYB109AS* transgenic plants, qRT-PCR analysis was performed. Total RNA was extracted from

ovules at 0 to 3 days post anthesis (DPA) of AS24-1, AS54-1, and the wild-type plants. The results showed that the level of *GhMYB109* mRNAs was reduced significantly (approximately eight fold) in the transgenic plants (Figure 2D). To check if the transgene also affected the expression level of other *GhMYB* genes, we further analyzed the expression levels of four *GhMYBs* (*GhMYB102a*, *GhMYB111*, *GhMYB139* and *GhMYB149*) (SUO *et al.* 2003) in ovules and fibers from the transgenic plants by qRT-PCR using the gene-specific primers (Table 1). There was no significant expression reduction of other *GhMYB* genes (Figure 2D). These results indicated that the expression levels of other MYB genes remained largely unchanged in both the transgenic plants and wild-type plants, showing that the antisense gene caused a gene-specific significant reduction in *GhMYB109* expression

Fiber development is impaired in the antisense transgenic plants: The transgenic plants showed a short-fiber phenotype indicating that the phenotype was a result of the knock-down of *GhMYB109* expression. Figure 3 shows the fiber development and seed phenotype of T_1 segregants. The impact of *GhMYB109* suppression on the cellular development of fiber initials was visualized using scanning electron microscopy. Fiber cells were differentiated and rapidly emerged from the surface of the ovule at 0 DPA in wild-type plants. Figure 3A shows the evenly arranged spherical fiber cells on the surface of wild-type ovules. By contrast, the fiber initials were much slower and smaller in AS54-1 ovules. Many of those cells were shrunken, and some had an abnormal shape and very weak projection above the ovule surface (Figure 3D). Similar shrunken fiber initials also were observed in

AS24-1. After initiation on 0 DPA, fiber cells in wild-type plants reached approximately 300 μm long at 3 DPA (Figures 3, B and C). This elongation process, however, was inhibited severely in the transgenic plants, and fibers were only less than 50 μm in length (Figures 3, E and F). In the stage of initiation, there were estimated 2,100±5.58 fiber cells per square millimeter from the ovule epidermis of the wild-type cotton, and 1930±5.87 fiber cells in AS54-1 ovules. This result suggested that an incomplete suppression of *GhMYB109* had a partial (approximately 8%) reduction of fiber initials, but it remains unclear if *GhMYB109* is directly involved in fiber initiation because the lack of a null allele. Measurement of the mature fiber length showed that the length of fiber in wild-type cotton reached 3.475±0.19 cm, whereas 2.3±0.12 cm in AS24-1 and 2.315±0.08 cm in AS54-1. Figure 3H shows the fiber length in the transgenic plants reduced approximately 33% compared with wild-type plants. Fiber elongation in the transgenic plants was slower than that in wild-type plants (Figure 3I). Most of the bolls of the transgenic plants were smaller than those in the wild type after maturation (Figure 3G), indicating that the *GhMYB109* antisense also slightly affected the boll development. The transgenic seeds could be germinated and grown, indicating that suppression of GhMYB109 only repressed the fiber development without affecting embryo development and viability.

To further examine the effect of the transgene, we analyzed the transgenic plants of T_2 generation (Figure 4). In the line AS54-1, one T_2 plant was obtained and had a single copy of the transgene as its parent (Figure 4A). For line AS24-1, among five

tested T_2 plants, four T_2 progeny had the transgene and retained the short-fiber phenotype, and one progeny without the transgene displayed a fiber phenotype similar to wild type (Figure 4B). The results suggested that the antisense gene was effective when it was in both the homozygous and hemizygous states. Taken together, these results indicated that *GhMYB109* plays a direct role in the elongation of cotton fiber cells.

Transcriptional reduction of several fiber-related genes in the transgenic plants: To examine possible targets of *GhMYB109* transcript reduction, we selected several known fiber-related genes, *GhACO1* and *GhACO2* (SHI *et al.* 2006), *GhTUB1* (LI *et al.* 2002), *GhACT1* and *GhACT5* (LI *et al.* 2005), for a comparative analysis between the transgenic and wild type cotton using qRT-PCR. Our results revealed that the *GhMYB109* suppression led to a substantial reduction of *GhACO1*, *GhACO2*, *GhTUB1* and *GhACT1* expression but had no apparent effect on the expression of *GhACT5* (Figure 5), indicating that *GhACO* and cytoskeleton-encoding genes likely represent potential downstream genes directly or indirectly regulated by *GhMYB109*.

DISCUSSION

Although the molecular mechanisms controlling cotton fiber initiation and elongation remain largely unknown, we have shown a direct role of the R2R3 MYB transcription factor *GhMYB109* in cotton fiber development. This was shown by its role in the knockdown of *GhMYB109* expression led to a substantial reduction in fiber length. This role also is consistent with its fiber-specific expression. To our

knowledge, GhMYB109 is among the first functional transcriptional factor which is directly implicated for cotton fiber formation.

Plant MYB genes have been shown to be involved in the regulation of many aspects of plant development, hormone signaling and metabolism. The MYB family is one of the largest groups of transcription factors in the Arabidopsis genome (KRANZ *et al.* 1998; STRACKE *et al.* 2001). Several MYB transcription factors, such as *GhMYB1-6*, *GaMYB2* and *GhMYB25*, have been identified in cotton. Although some of them have been characterized with fiber-specific expression, their roles in the cotton fiber development are not yet well defined. The role of *GhMYB109* is consistent with its highly conserved R2R3 MYB domain. From previous studies it is clear that many proteins with the similar R2R3 MYB factors are involved in the control of development and determination of cell fate and identity (RAMSAY and GLOVER 2005; SCHIEFELBEIN 2003). The role of MYB transcriptional regulators in trichome formation extends beyond Arabidopsis and cotton. A R2R3 MYB-related transcriptional factor MIXTA regulates the formation of conical shape in petal epidermal cells of snapdragon (*Antirrhinum majus*) (GLOVER *et al.* 1998; MARTIN *et al.* 2002; NODA *et al.* 1994). In *Petunia hybrida*, conical cell formation in the petals also requires a MYB-related transcription factor named PhMYB1, which is structurally related to MIXTA (AVILA *et al.* 1993; VAN HOUWELINGEN *et al.* 1998). The *MYB MIXTA LIKE 1* (*AmMYBML1*) gene from *A. majus* encodes an R2R3 MYB-related transcriptional regulator identical to that of MIXTA, and also promotes trichome and conical cell formation on floral tissues when it was overexpressed under

the control of the 35S promoter in tobacco (GLOVER *et al.* 1998; MARTIN *et al.* 2002; PEREZ-RODRIGUEZ *et al.* 2005). In light of these analyses, our study provides a remarkable example of the essential role of MYB transcription factor in plant growth at the level of a single cell. Because of our findings, we hypothesize that unicellular or multicellular plant hairs develop likely through a similar network of transcription factors (or transcriptional cassette), revealing a functional conservation in cell fate determination in plants.

We have shown that knockdowns of *GhMYB109* dramatically reduce cotton fiber elongation, but it remains unclear how the transcription factor controls fiber cell development. In Arabidopsis, AtGL1/AtWER physically interacts with the bHLH proteins AtGL3/AtEGL3 to regulate transcription as part of a multi-protein complex which promotes trichome or root hair cell fate determination (RAMSAY and GLOVER 2005; SCHIEFELBEIN 2003; SERNA and MARTIN 2006). The complex of MYB-bHLH-WD40 appears to regulate the trichome-specific expression of *GL2*, an activator of downstream trichome–specific differentiation genes, whereas *TRY* (*CPC* or *ETC1*) is a negative regulator that represses trichome differentiation by competing with the MYB factors for binding of the initiation complex (SERNA and MARTIN 2006). It is possible that similar transcription factors in cotton bind to target genes that are involved in the transcriptional regulation of fiber development.

We have found that the *GhMYB109* suppression induced the expressional reduction of GhACO1, GhACO2 (SHI *et al.* 2006), GhTUB1 (LI *et al.* 2002) and GhACT1 (LI *et al.* 2005) (Figure 5). These results indicated that the MYB-regulated

genes are induced prior to the phytohormonal pathway or cytoskeleton related genes, suggesting that the transcription factor likely regulates these genes for cell fate determination. We hypothesize that the activity of cotton MYB genes is involved in regulating the fiber cell development just at the stage of initiation. When fiber cells begin to enlarge and elongate rapidly at the stage of primary cell wall formation, the transcription factors activate the transcriptions of the phytohormonal pathway (*GhACOs* or other related genes), cytoskeleton (*GhTUBs* and *GhACTs*) or other fiber-related genes to elaborate and maintain the rapid fiber growth. It is worth examining whether some MYB-binding site elements occur in promoters of *GhACOs* or cytoskeleton genes. In addition, the cotton homologs related to *MIXTA*, *MYB5* and *GL2* are activated during fiber cell initiation (YANG *et al.* 2006). Wang *et al.* have shown that two cotton transcription factors, GaMYB2/Fiber Factor 1 (FIF1) and GhHOX3, are able to activate the promoter of a cotton fiber gene, *RD22-like1* (*RDL1*) (WANG *et al.* 2004b). However, it remains to be seen how these genes are regulated and whether this regulation is directly or indirectly related to cotton fiber development.

In conclusion, the results of this study contribute to an understanding of the developmental mechanism of fiber development and provide the direct evidence that *GhMYB109* is required for the development of single-celled fibers of cotton. With the demonstration of a fiber-specific promoter from *GhMYB109*, we will able to express target gene products in the developing fiber for possible genetic improvement of fiber development.

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FIGURE LEGENDS

Figure 1.—Histochemical localization of GUS activity in the transgenic cotton with the *GhMYB109::GUS* fusion gene. (A) A schematic representation of the *GhMYB109 Promoter::GUS* fusion construct used for cotton transformation. (B) and (C) Dark-field micrographs of 8-μm-thick longitudinal (B) and cross(C) sections of

3DPA ovules. A high level of GUS activity represented by pink dots was only found in the fiber cells. f, fiber; e, epidermis; o, outer integument of ovule. (D) to (H) Bright field of micrographs and photographs of ovules and other tissues in the transgenic and nontransformed plants.(D) to (F) GUS staining in ovules at 3DPA. No GUS staining was detected in the ovules of the nontransformed cotton (D). Strong GUS activity was observed in the fibers of the transgenic plants (E) and (F). (F) A longitudinal section of a transgenic ovule. (G) GUS staining in each stage of transgenic cotton bolls, 1DPA, 3DPA and 5DPA (from left to right). The left two are longitudinal sections of cotton bolls. (H) GUS staining in other tissues of the transgenic cotton. No GUS activity was detected in leaf, sepal, stem and flower bud before anthesis (from left to right). Bars, 100μm in (A) and (B)；1mm in (D), (E) and (F); 2mm in (G); 1cm in (H).

Figure 2.—Molecular analysis of the antisense *GhMYB109* transgenic cotton. (A) A schematic representation of the antisense *GhMYB109* construct used for cotton transformation. (B) and (C) DNA gel blot analysis of the transgenic lines. Genomic DNA (20μg/lane) of two independent transgenic (AS24-1/2 and AS54-1) and wild-type plants were digested with *Eco*RI and *Hin*dIII, respectively, transferred to nylon membrane and hybridized with ^{32}P-labeled *NPTII* (B) and ^{32}P-labeled *GhMYB109* (C). (D) Quantitative real-time PCR analysis of the transgenic lines. Total RNA were isolated from 0DPA, 1DPA and 3DPA ovules with their fibers attached of AS24-1, AS54-1 and wild-type plants and subjected to quantitative real-time PCR analysis (qRT-PCR) using *GhMYB109*, *GhMYB111, GhMYB139, GhMYB149* and

GhMYB112a-specific gene primers, respectively, and 18S rRNA as an internal control to normalize all data. The *GhMYB109* expression was significantly reduced in the transgenic plants, whereas the expression of the other *GhMYB* genes was barely affected in the transgenic lines.

Figure 3.—Comparison of the fiber initiation and length between the antisense transgenic *GhMYB109* and wild-type cotton. (A) to (F). Scanning electron micrographs of ovule surface of the antisense transgenic *GhMYB109* (AS54-1) and wild-type plants. Ovules of the wild-type and transgenic plants are at 0DPA (A and D), 3DPA (B and E) and (C and F). The length of fibers in the transgenic plant is much shorter than that in wild-type plant at the same stage. (G) Mature bolls from the transgenic plant AS24-1 and AS54-1 were smaller than that in the wild type. (H) Fibers in the transgenic plant AS24-1 and AS54-1 were much shorter than that in the wild type. (I) Mature fiber lengths of the transgenic antisense *GhMYB109* and wild-type cotton seeds. Measurement of the fiber lengths showed that the fiber length in the transgenic plants was reduced approximately 33% compared with wild-type plants. f: fiber; s: stoma. Bars: 2cm in (G) and 1cm in (H).

Figure 4.—Examples of the transgene copy number testing and mature fiber length of the T_2 cotton transgenic progeny. (A) Genomic DNA (20μg/lane) of the wild-type and the T_2 of the two independent transgenic (AS24-1 and AS54-1) plants were digested with *Eco*RI (left) and *Hin*dIII (right), respectively, transferred to nylon

membrane and hybridized with ^{32}P-labeled *NPTII*. From left lane: WT, Wild type plant; 1- 5, five T_2 progeny of AS24-1; 6, one T_2 progeny of AS54-1. Molecular weight markers are indicated in kilo basepairs. (B) Mature fiber lengths of the T_2 cotton transformants and wild-type cotton seeds. Measurement of the fiber lengths showed that fiber elongation in the transgenic plants was shorter than that in wild type plant. AS24-1-2, one T_2 plant of AS24-1 without the transgene copy, displayed a fiber phenotype similar to wild type**.**

Figure 5.—Expression profiling of seven genes important for fiber development in the wild-type and the transgenic plants. Total RNA samples prepared from 0DPA, 1DPA and 3DPA ovules with their fibers attached of the two *GhMYB109* antisense transgenic and wild-type plants were used for qRT-PCR analysis. 18S rRNA was used as an internal control. The expression of *GhACT5* gene appeared not to be affected in the transgenic lines, whereas *GhACO1*, *GhACO2*, *GhTUB1* and *GhACT1* were expressed at lower levels in the transgenic plant than that in wild-type.

Table 1. Primers used for real-time PCR analysis.

Genes	Primers
GhMYB109	5'-AAGAAGGTGAAATTCTATACAAAAAGG-3'(forward)
	5'-TCCATGGACATTGACATAATCA-3'(reverse)
GhMYB102a	5'-CATGTGGGGGAGAAAGAAGA-3'(forward)
	5'-TGAGGCTGTCAAAACTGCTG-3'(reverse)
GhMYB111	5'-GCAAACCCAACCAGAGTCAT-3'(forward)
	5'-GGTGCTGCAAGTGCAATCT -3'(reverse)
GhMYB139	5'-AAACCTGACCCTGACTTTTTCCT -3'(forward)
	5'-TCGATTTCCGAAACGATTCC-3'(reverse)
GhMYB149	5'-GGGTCCGATTTGAGCGATT-3'(forward)
	5'-GGGCTTGTACACCGTGTGAA-3'(reverse)
GhACO1	5'-CTGACAAATCTCAAGTGTACCCC-3' (forward)
	5'-AAGTTAACTGCAGACTCCACG -3'(reverse)
GhACO2	5'-CCCTAAACCCGACCTAATCA-3'(forward)
	5'-AGGAGTTGAAGCCCACTGAC-3'(reverse)
GhACT1	5'- GGAGACTGGATTGTGGTGCTT-3'(forward)
	5'- CGCGCAAACTGGGACTAACT-3'(reverse)
GhACT5	5'-CTCTGAAGCTCCTCTTGGTTC-3'(forward)
	5'-TATCACAGACGAGGGGTTGA-3'(reverse)
GhTUB1	5'-CGGTACCATGGATAGCGTAA-3'(forward)
	5'-TCCCTTAGCCCAATTGTTTC-3'(reverse)
18S rRNA	5'-CGGCTACCACATCCAAGGAA-3'(forward)
	5'-TGTCACTACCTCCCCGTGTCA-3'(reverse)

Figure 1.

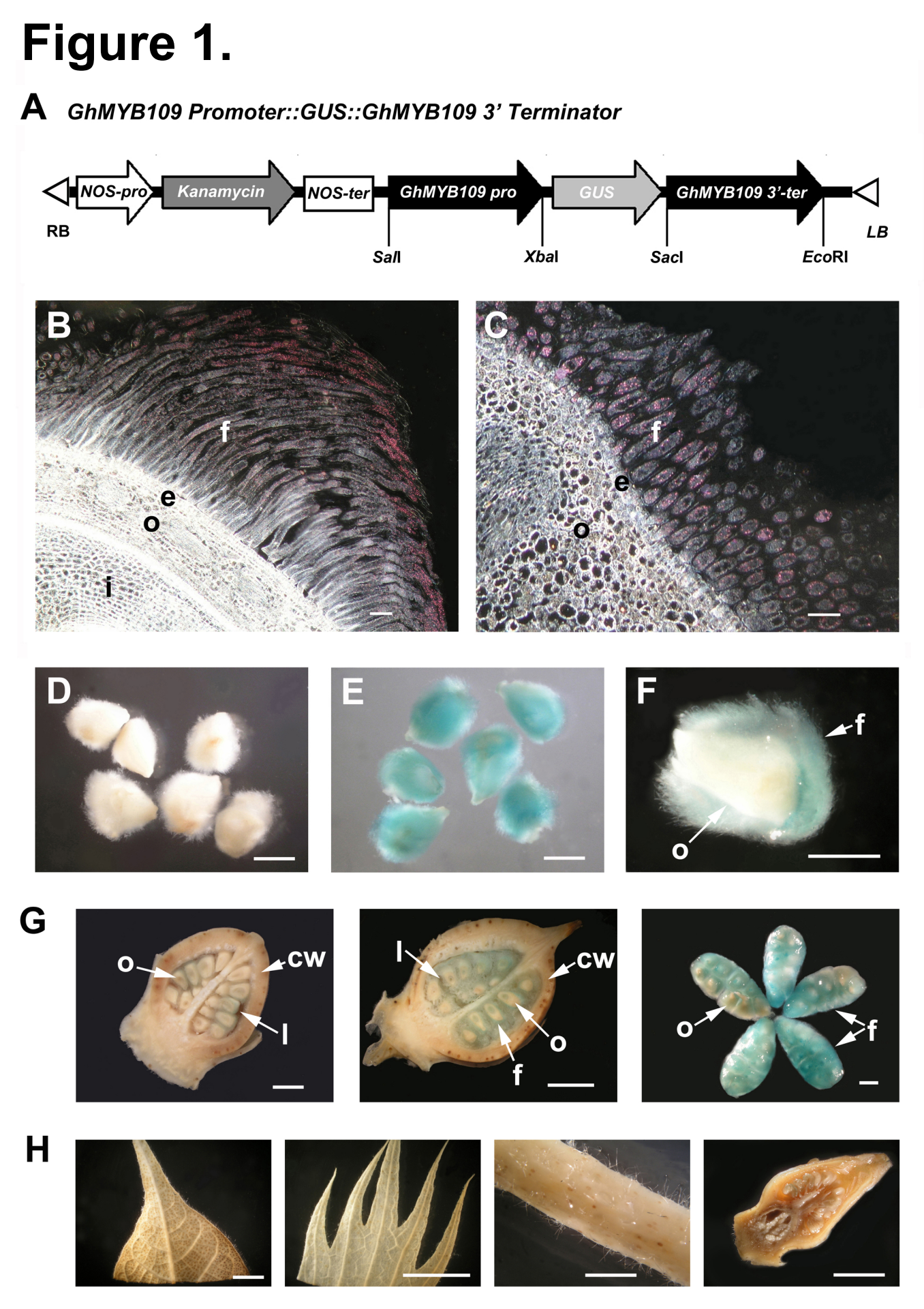

A
GhMYB109 Promoter::GUS::GhMYB109 3' Terminator
NOS-pro
Kanamycin
NOS-ter
GhMYB109 pro
GUS
GhMYB109 3'-ter
RB
LB
SalI
XbaI
SacI
EcoRI
B
f
e
o
i
C
f
e
o
D
E
F
f
o
G
o
cw
l
l
cw
o
f
o
f
H

Figure 2.

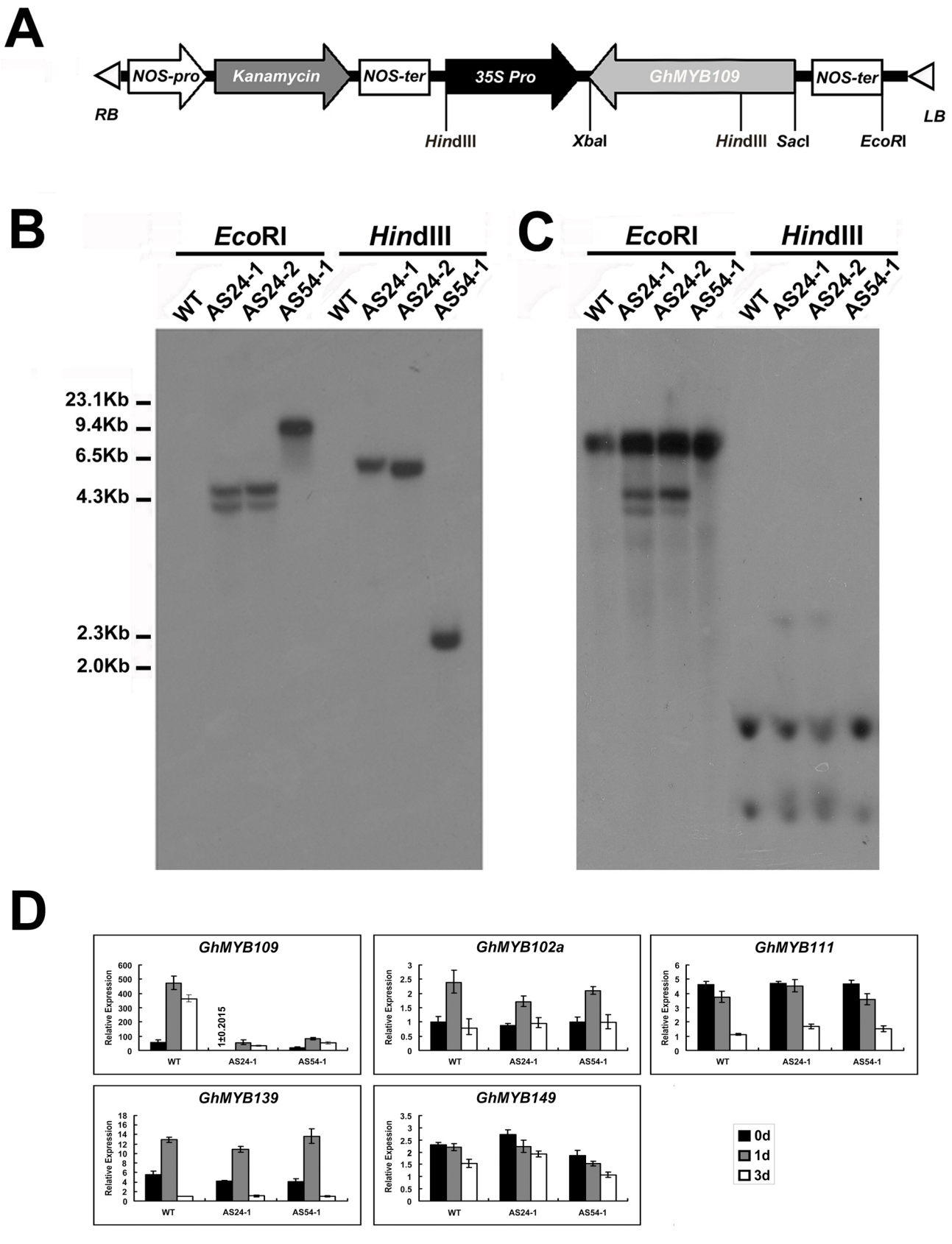

A
NOS-pro
Kanamycin
NOS-ter
35S Pro
GhMYB109
NOS-ter
RB
LB
HindIII
XbaI
HindIII
SacI
EcoRI
B
EcoRI
HindIII
WT
AS24-1
AS24-2
AS54-1
23.1Kb
9.4Kb
6.5Kb
4.3Kb
2.3Kb
2.0Kb
C
EcoRI
HindIII
WT
AS24-1
AS24-2
AS54-1
D
GhMYB109
GhMYB102a
GhMYB111
GhMYB139
GhMYB149
Relative Expression
1±0.2015
WT
AS24-1
AS54-1
0d
1d
3d

Figure 3.

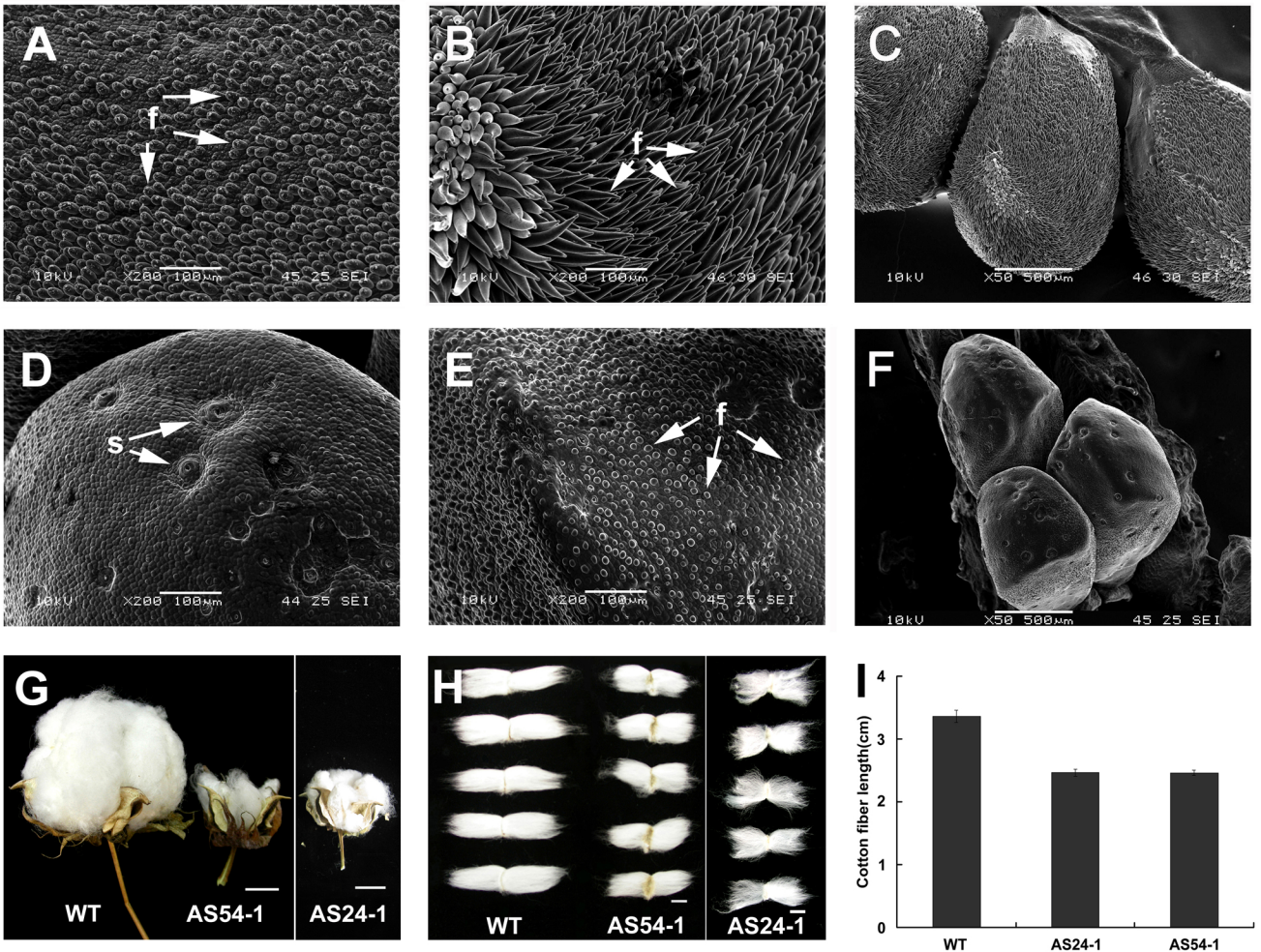

A
f
10kV X200 100µm 45 25 SEI
B
f
10kV X200 100µm 46 30 SEI
C
10kV X50 500µm 46 30 SEI
D
s
10kV X200 100µm 44 25 SEI
E
f
10kV X200 100µm 45 25 SEI
F
10kV X50 500µm 45 25 SEI
G
WT
AS54-1
AS24-1
H
WT
AS54-1
AS24-1
Cotton fiber length(cm)
0
1
2
3
4
WT
AS24-1
AS54-1

Figure 4.

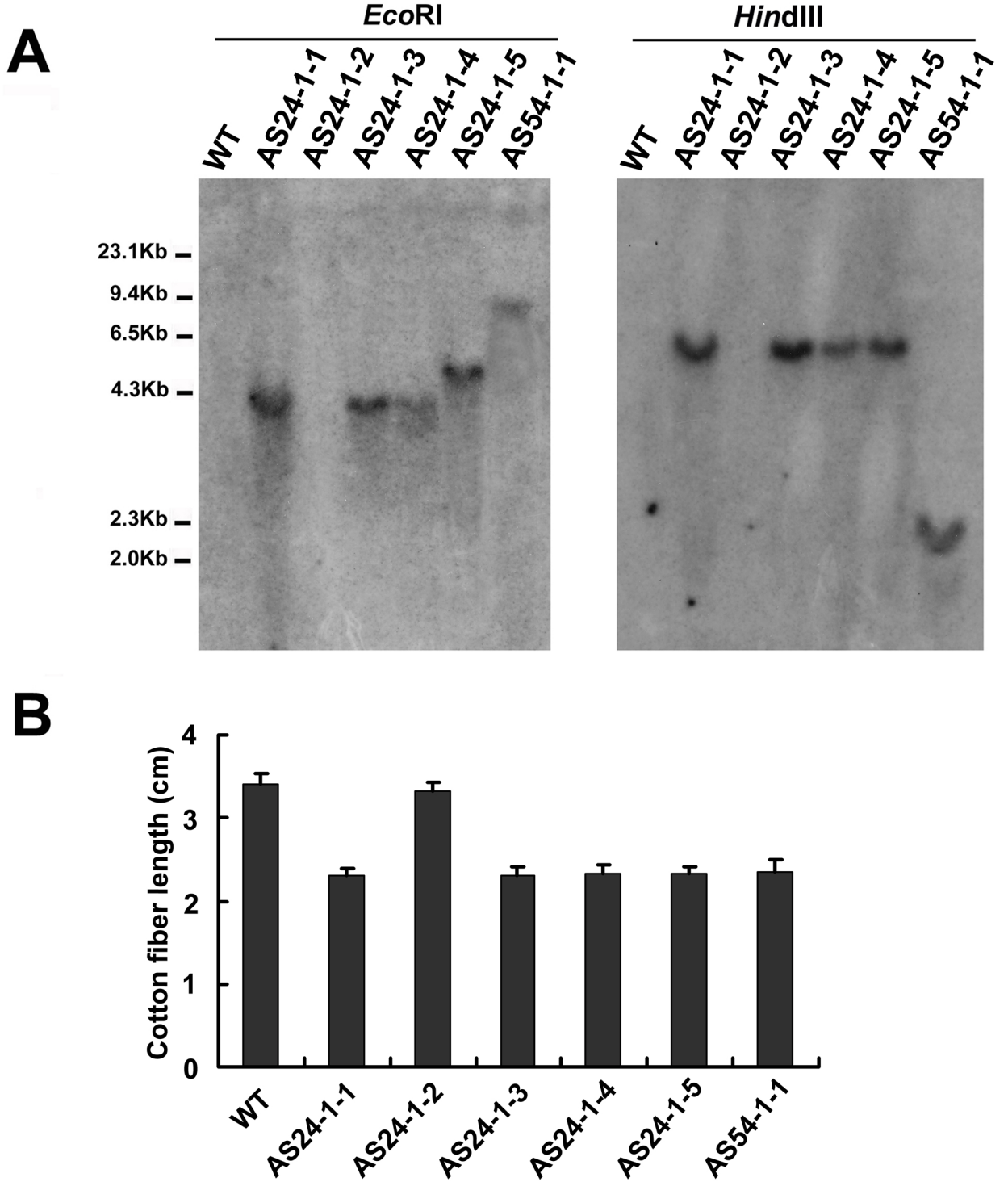
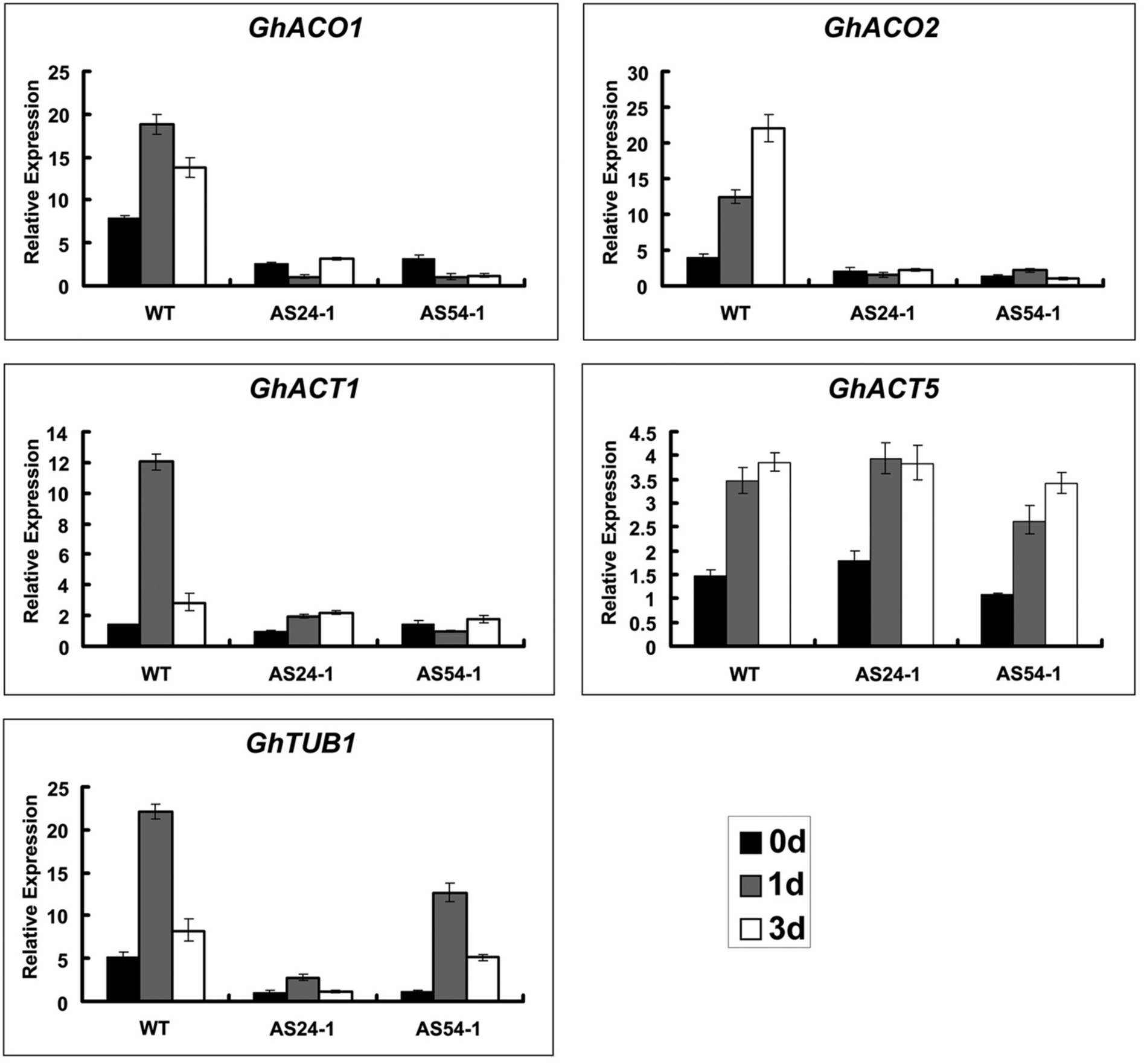

A
EcoRI
HindIII
WT
AS24-1-1
AS24-1-2
AS24-1-3
AS24-1-4
AS24-1-5
AS54-1-1
23.1Kb
9.4Kb
6.5Kb
4.3Kb
2.3Kb
2.0Kb
B
Cotton fiber length (cm)
0
1
2
3
4
WT
AS24-1-1
AS24-1-2
AS24-1-3
AS24-1-4
AS24-1-5
AS54-1-1

Figure 5.


GhACO1
Relative Expression
25
20
15
10
5
0
WT
AS24-1
AS54-1
GhACO2
Relative Expression
30
25
20
15
10
5
0
WT
AS24-1
AS54-1
GhACT1
Relative Expression
14
12
10
8
6
4
2
0
WT
AS24-1
AS54-1
GhACT5
Relative Expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
AS24-1
AS54-1
GhTUB1
Relative Expression
25
20
15
10
5
0
WT
AS24-1
AS54-1
0d
1d
3d