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Isolation of *GhMYB9* gene promoter and characterization of its activity in transgenic cotton

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Abstract

The *GhMYB9* encodes a R2R3 MYB transcription factor in the upland cotton (*Gossypium hirsutum* L.) genome. Our studies show that *GhMYB9* predominantly expressed in flowers and fibers. To gain a better understanding of its regulatory mechanism, we isolated the 5'-flanking region of *GhMYB9* which was 1 487 bp in length. The *cis*-acting element prediction shows that this region contained the basic structure of the core promoter elements (TATA-box, CAAT-box) and the transcription start site (TSS). Other motifs, such as defense and stress responsiveness (TC-rich repeats), anaerobic induction (ARE), and MYB binding sites involved in drought-inducibility (MBS), were also found. Histochemical assay shows that the *GhMYB9* promoter governed β-glucuronidase (GUS) expression mainly in seeds, fibers, and flowers of transgenic cotton. Also, the activity of the promoter was induced by auxin in fibers of transgenic cotton. This is consistent with its transcript abundance in different tissues. A further deletion analysis confirms that a promoter region from -1 231 to -860 was required for auxin response. Our findings provide a useful reference for the understanding of the transcriptional regulation mechanism of the *GhMYB9* gene.

Additional key words: auxin, gene expression, GUS staining.

Introduction

As one of the largest transcription factor families in higher plants, the R2R3-MYB transcription factors are key factors in regulating many aspects of plant development, hormone signaling, metabolism, and responses to biotic and abiotic stresses (Jin and Martin 1999, Dubos *et al*. 2010). Many reports have revealed that particular members of the R2R3-MYB class act as positive regulators in epidermal cell differentiation. A mutation of a R2R3-MYB gene *GLABRA1* (*GL1*) results in glabrous plants in *A. thaliana* (Hauser *et al*. 2001). Cotton fiber shares many similarities with *A. thaliana* leaf trichome development. In cotton, many *MYB* genes have been identified *via* molecular studies. These MYB transcription factors were proved to be responsible for fiber development, namely *GhMYB109* (Pu *et al*. 2008), *GhMYB2* (Wang *et al*. 2004, Guan *et al*. 2014), *GhMYB25* (Machado *et al*. 2009), *GhMYB25*-like (Walford *et al*. 2011), *etc*. Besides a function in cell differentiation, some MYB transcription factors play roles in anther forming and pollen development. They specifically express in floral organs and are involved in forming the tapetal layer (Yang *et al*. 2001, Higginson *et al*. 2003, Steiner-Lange *et al*. 2003), the dynamic change of callose (Zhang *et al*. 2007, Zhu *et al*. 2008), and anther dehiscence during pollen development (Song *et al*. 2011, Li *et al*. 2013). In allotetraploid cotton, there are over 200 MYB transcription factors (Paterson *et al*. 2012), among which biological functions are largely unknown.

The *GhMYB7* and *GhMYB9* are homoeologous genes in the allotetraploid cotton genome, encoding R2R3-MYB transcription factors. Previous studies using Northern blot analysis demonstrated that *GhMYB7/9* predominantly express in flowers and fibers of cotton and

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Abbreviations: ARE - anaerobic induction; DPA - days post-anthesis; GA_3 - gibberellic acid; GUS - β-glucuronidase; IAA - indole-acetic acid; MBS - MYB binding sites involved in drought-inducibility; MUG - 4-methylumbelliferone; SNP - single nucleotide polymorphism.

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are induced by auxin treatment (Hsu *et al.* 2005). To better understand the role and regulation of *GhMYB9*, we isolated and analyzed the promoter of this gene and probed its function based on expression of the β-glucuronidase (*GUS*) reporter gene driven by the *GhMYB9* promoter in transgenic cotton. Here, we followed promoter activity in young roots, fibers, pollens, and ovaries, and also its possible induction by auxin and compared it with *GhMYB9* transcript abundance in these tissues.

Materials and methods

Plant growth and transformation: Upland cotton, *Gossypium hirsutum* L. cv. TM-1, accession W0 and transgenic cotton plants were grown in a greenhouse facility in the Nanjing Agricultural University in China. The growing conditions were: a constant temperature of 28 °C, a relative humidity of 70 %, a 16-h photoperiod, and an irradiance of 500 μmol m^{-2} s^{-1}.

Agrobacterium-mediated cotton transformation was performed as described Li *et al.* (2002). After induction, differentiation, and plantlet regeneration, the plantlets were grafted onto rootstock *Gossypium barbadense* L. cv. H7124 and grown in a greenhouse. Kanamycin at 2000 mg dm^{-3} was used and leaves of the kanamycin susceptible seedlings will change color from green to yellow after 5 - 7 d. After 3 repeated applications, kanamycin-resistant plants were kept for further molecular analysis. After self-pollination, T1 generation seeds were collected, and self-pollinated T2 and T3 generation seeds were harvested over the next 2 years.

Quantitative RT-PCR: Total RNA from various organs was isolated using the cetyl-trimethyl ammonium bromide (CTAB) method (Gambino *et al.* 2008), and 2 μg of total RNA was used for reverse transcription. According to the single nucleotide polymorphism (SNP) site between the exons of the *GhMYB7* (GeneBank: AY518319) and *GhMYB9* (GeneBank: AF336286) genes, primers designed using *WebSNAPER* (http://pga.mgh.harvard.edu/cgi-bin/snap3/ websnaper3.cgi), namely W319 and W321 (Table 1), were used to detect the expressions of *GhMYB7* and *GhMYB9*. The *EF-1α* (Y8991F\R; Table 1) from cotton was used as an internal control for normalization of different cDNA samples. PCR was performed using a real-time PCR system (*Bio-Rad*, Hercules, USA) along with a *SYBR Green PCR Master* mix (*Applied Biosystems*, Foster City, USA). All PCRs were repeated three times, and the data were evaluated using the comparative cycle threshold method described by Livak and Schmittgen (2001).

Vector construction: The TAIL-PCR was employed to isolate the 5'-flanking regions of *GhMYB9*, and a 1 487 bp sequence upstream of the translation start site (TSS) of *GhMYB9* was obtained. Based on the structural characteristics and distribution of *cis*-acting elements in the *GhMYB9* promoter predicted by *plantCARE* (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), three promoter truncates, namely *pF1* (from +122 to -1 231 nt), *pF2* (from +122 to -860 nt), and *pF3* (from +122 to -268 nt), were amplified using a combination of forward primers Y2338, Y2339, and Y2340, respectively, and a reverse primer Y2341. To facilitate vector construction, a *Sbf*I site was introduced into the 5' ends of Y2338 and Y2339, a *Hin*dIII site was introduced into the 5' end of Y2340, and an *Xma*I site was introduced into the 5' end of the reverse primer Y2341. Three PCR products were cloned into a *pGEM-T easy* vector (*Promega*, Madison, USA). Each truncated product was verified by sequencing. Then, replacing the CaMV35S promoter in the *pBI121* vector directly, three promoter fractions were fused to the GUS-coding gene to produce three plant expression vectors, *pF1*, *pF2*, and *pF3*. All primer sequences used in TAIL-PCR and the vector construction are listed in Table 1 Suppl.

Histochemical staining and fluorometric quantification of GUS activity: Histochemical localization of GUS was performed according to the method of Jefferson (1987). Photography was performed with a stereomicroscope (*DP72, Olympus*, Tokyo, Japan) or a fluorescence microscope (*DM2500, Leica*, Solms, Germany).

Total proteins were extracted by grinding a frozen plant tissue in an extraction buffer consisting of 50 mM sodium phosphate (pH 7.0), 0.1 % (v/v) *Triton X-100*, 10 mM Na_2EDTA, 0.1 % (m/v) sodium lauryl sarcosine, and 10 mM β-mercaptoethanol. The supernatant was then collected after centrifugation at 10 000 *g* and 4 °C for 10 min. The total protein content was determined using the Bradford (1976) method. A fluorogenic substrate 4-methylumbelliferone (MUG) was used for fluorometric quantification of GUS activity (Jefferson *et al.* 1987). The GUS activity was calculated as pmol of MUG per mg protein per min.

Effect of auxin on *GhMYB9* promoter: To test whether the activity of *GhMYB9* promoter was induced by auxin, an *in vitro* cotton ovule culture system described by Beasley (1971) was used. The ovules of the transgenic plants at 0 days post-anthesis (DPA), harboring three truncated *GhMYB9* promoters, were cultured for 10 d in a medium containing 2 μM indole-acetic acid (IAA) and 2 μM gibberellic acid (GA_3). Then, the ovules were moved on a medium containing 10 μM IAA and 2 μM GA_3 for 2 d (Hsu *et al.* 2005). All fibers detached from the cultured ovules were collected for RNA extraction and GUS activity measurement by the methods described before.

Results

The temporal and spatial expression patters of *GhMYB9* in fiber developmental stages and different tissues were examined by RT-qPCR. Our results show that *GhMYB9* predominantly expressed in cotton flowers and fibers. During fiber development, the expression of *GhMYB9* peaked at 10 DPA and gradually decreased after that. The *GhMYB7* shared a similar expression pattern with *GhMYB9,* but at a relatively lower expression (Fig. 1).

Seven arbitrary degenerate primers (Y661 - Y667) were tested in combination with a set of nested primers (Y2072, Y2073, and Y2074). Each primer combination resulted in the amplification of discrete PCR products ranging from approximately 200 to 2 000 bp (Fig. 2). We recovered a tertiary PCR product of approximately 800 bp in length using the Y666 primer; it was then sequenced. This PCR product overlapped perfectly with the 5'-end sequence of the *GhMYB9* gene. Finally, through sequences assembly, the 1 487-bp fragment upstream of the translation start site (ATG) of *GhMYB9* was obtained as promoter. According to the analysis of the promoter sequence, the transcription of *GhMYB9* started at the A within the TC$\underline{\text{A}}$TACT sequence,

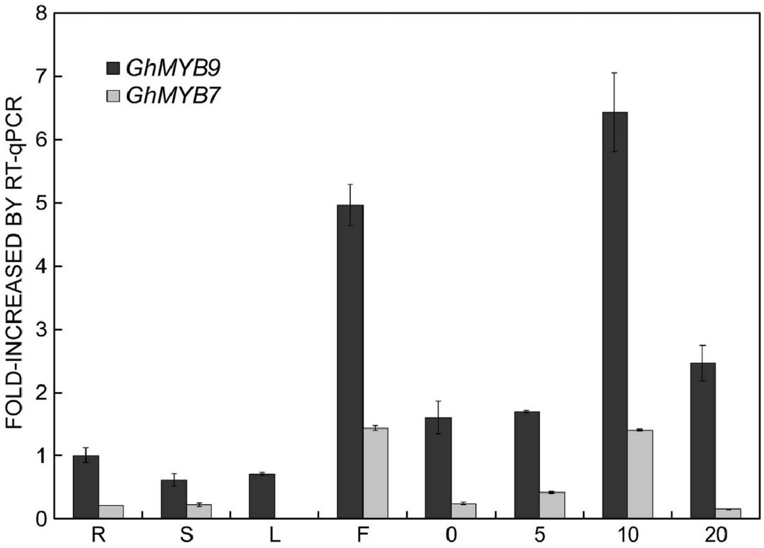

Fig. 1. The expression of *GhMYB*9 and *GhMYB*7 in cotton. R - roots, S - stems, L - leaves, 0 - 0 DPA ovules, 5 to 20 - 5 to 20 DPA fibers. Fold increases relative to the roots value of *GhMYB*9 (arbitrarily set to 1.0). *Error bars* represent SE of means based on three repeated reactions.

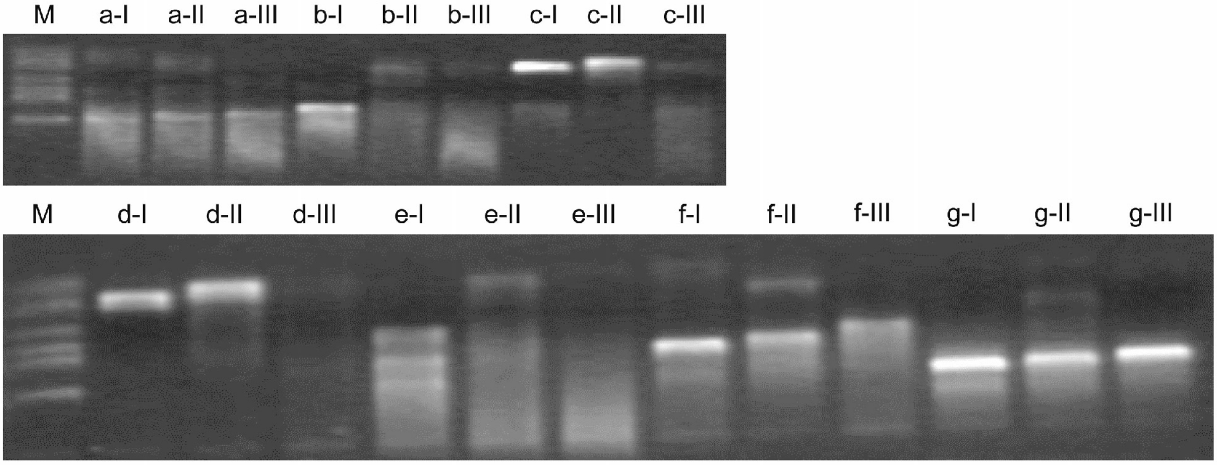

Fig. 2. The agarose gel analysis of TAIL-PCR products. Each set of three lanes contained products from secondary and tertiary reactions, and a single AD primer amplification: a to g indicate the combination of different AD primers (Y661 - Y667) with a set of specific primers. I is the secondary PCR product, II is the tertiary PCR product, and III is the amplified fragment from AD primers using the secondary PCR product as template. *Lane* M: *DL2000* markers.

Fig. 3. The nucleotide sequence of the *GhMYB9* promoter. The 1 487 bp upstream region of the *GhMYB9* promoter from the initiation site is shown. The location +1 is the putative transcription site, and the TATA box and the CAAT box are *underlined*. The forward primers and the common reverse primer of F1, F2, and F3 fusions are in *bold* and are indicated by the *arrows*. All putative *cis*-acting elements except radiation-response elements are *boxed*.

which perfectly matched the consensus sequence (PyPyANT/APyPy, Py:C/T, and N:A/T/C/G) for plant transcription start sites. The first nt (A) of the transcription start site (TSS) was assigned as position +1 in the sequence, and the positions upstream of position +1 are indicated with negative numbers. By searching with *PlantCARE*, regulatory elements including the TATA box and the CAAT box and the transcription start site were found upstream of the initiation codon (Fig. 3 and Table 2). Besides core motifs, other elements were mainly divided into three classes. The first class concerned radiation responses and comprised 12 elements and motifs (AE-box, Box I, Box II, Box4, CG-motif, G-box, GAG- motif, GT1-motif, PcCMA2a, Sp1,

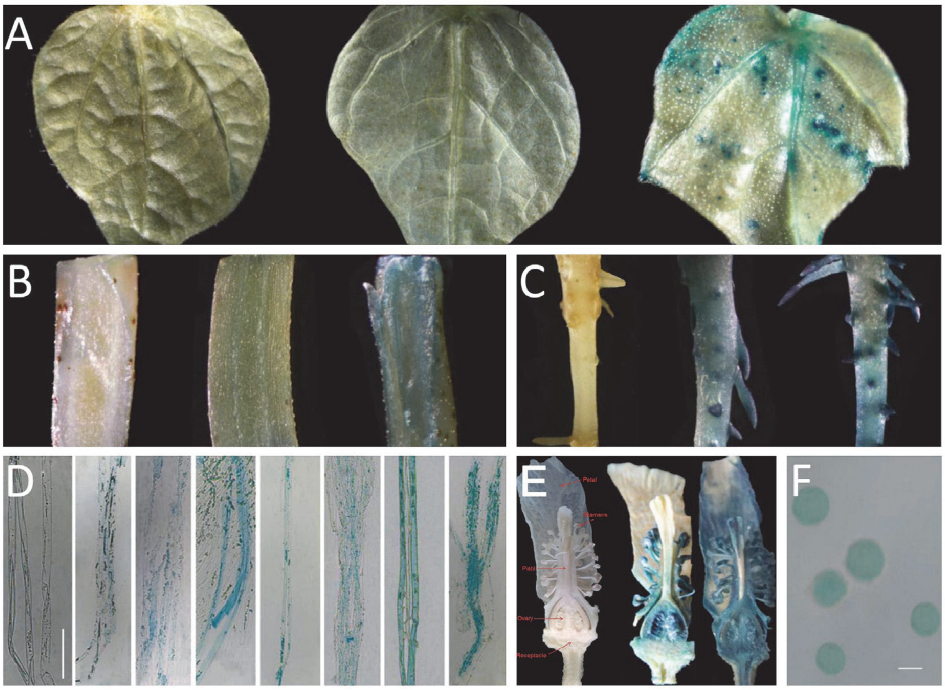

Fig. 4. *In situ* localization of GUS staining in leaves (*A*), stems (*B*), roots (*C*), different developmental stages of fibers (*D*), flower organs (*E*), and pollen (*F*). In *A*, *B*, *C*, and *E*, from left to right, there are presented a non-transgenic plant, a plant transformed with pF1-GUS, and a plant transformed with pBI121, respectively. In *D*, from left to right, it sequentially represents 10 DPA fiber of non-transgenic plant, 5, 8, 10, 14, 17, and 20 DPA fibers of pF1-GUS transgenic plants, and pBI121 transgenic plant, respectively. *F* shows GUS staining in the pollen of a pF1-GUS transgenic plant. The *scale bar* is 100 μm.

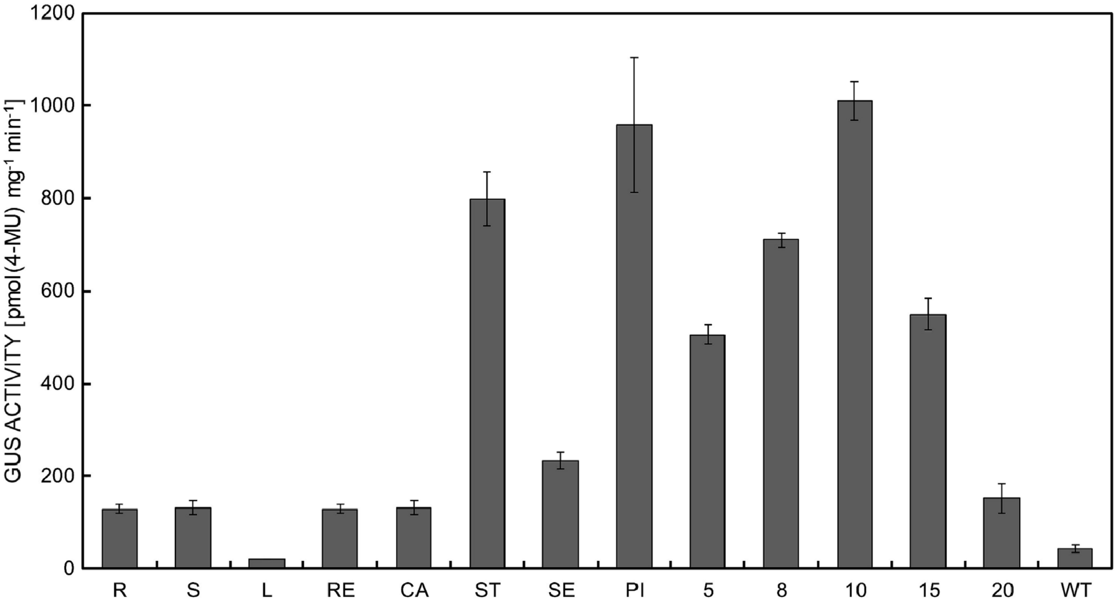

Fig. 5. Quantitative GUS assays of the *GhMYB9* promoter. R - roots, S - stems, L - leaves, RE - receptacles, CA - calycles, ST - stamens, SE - sepals, PI - pistils, 5 to 20 - 5 to 20 DPA fibers, WT - 10 DPA fibers of transgenic receptors W0. Means ± SE, $n = 3$.

rbcS-CMA2a, and TCT-motif). The second class concerned mainly a binding L1 box and four MYB binding sites (MBSs).

To study the characteristics of the *GhMYB9* promoter, we tested the promoter-driven GUS staining and activity in various tissues and organs in the *pF1-GUS* transgenic cotton. A high GUS expression was found in young root tips (Fig. 4*C*), fibers (Fig. 4*D*), and flower organs (Fig. 4*E*,*F*). Very weak staining was observed in leaves and stems (Fig. 4*A*,*B*). It is consistent with the GUS activity measurement (Fig. 5). During the fiber development stages, GUS activity was elevated already at 5 DPA and reached a peak at 10 DPA following by a gradual decline until 20 DPA (Fig. 4*D* and Fig. 5). In floral tissues, very strong staining was detected in stamens and ovaries, whereas weak staining was observed in receptacles and stigmas (Fig. 4*E* and Fig. 5). This is consistent with the *GhMYB9* gene expression pattern in different tissues and during fiber development. No GUS activity was detected in the non-transgenic controls.

We examined GUS activity governed by three truncated promoters in ovules of the transgenic plants under the IAA treatment. In fibers of the *pF1-GUS* transgenic plants, the GUS activity significantly increased by the IAA treatment; it increased 1.5-fold compared with WT implying that the cloned *GhMYB9* promoter (-1 231 to +122) contained a motif for auxin response. No significant changes were observed in the *pF2-GUS* or *pF3-GUS* transgenic lines suggesting that -860 to +122 nt and -524 to +122 nt regions had no function in response to auxin (Fig. 6). Taken together, these results suggest that the -1 231 to -860 nt region may be essential for this gene response to auxin.

Overall, our data suggest that the *GhMYB9* promoter cloned in this study was a tissue specific promoter that is specifically expressed in fibers and flowers. In addition, the 1 487 bp promoter sequence contained structures responsible for the auxin response of *GhMYB9*.

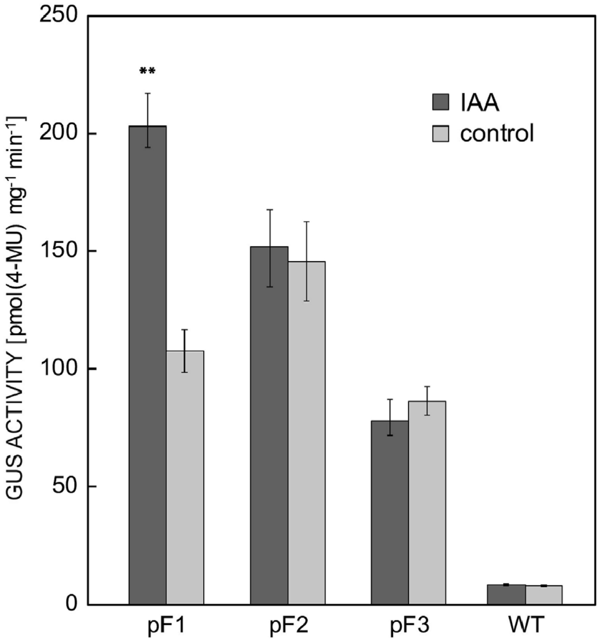

Fig. 6. The effect of IAA on GUS activity in fibers of transgenic cotton. The pF1, pF2, and pF3 are three truncates. Ovules were treated with 2 μM GA_3 and 10 μM IAA. Ovules treated with 2 μM GA_3 and 2 μM IAA served as control. Means ± SE, *n* = 3. The *double asterisks* mark a significant difference at the 0.01 level of probability.

Discussion

The R2R3-MYB transcription factors regulate diverse processes including organ development, secondary metabolism, and stress responses (Du *et al.* 2009, Dubos *et al.* 2010). Hsu *et al.* (2005) found that *GhMYB7* and *GhMYB9*, which belong to R2R3-MYB transcription factors, are homeologous/alloallelic genes in allotetraploid cotton (AADD), and *GhMYB7/9* predominantly expressed in flowers and fibers. Two homeologous genes may differentially express owing to genome merger and doubling in allotetraploid cotton (Cedroni *et al.* 2003, Flagel and Wendel 2010). It is difficult to distinguish the expression between the two homeologous genes due to their sequence similarity. Here, SNP primers were used to discriminate expression between *GhMYB7* and *GhMYB9*. Our results show that *GhMYB7* and *GhMYB9* had similar but biased expression patterns. To study the *GhMYB9* expression in detail, a 1 487 bp promoter was cloned and analyzed in cotton. In the *pF1-GUS* transgenic plants, the GUS activity in stamens and pistils was higher than in roots, stems, or sepals. Similarly, GUS staining was also very strong in stamens and pistils, and especially in filaments, anthers, and ovaries. In plants, R2R3-MYB transcription factors which play an important role in filament and anther development has been reported (Yang *et al.* 2001, Higginson *et al.* 2003, Steiner-Lange *et al.* 2003, Zhang *et al.* 2007, Zhu *et al.* 2008, Song *et al.* 2011, Li *et al.* 2013). The *GhMYB9* and *AtMYB26* have a 97.4 % homology in N-termini (DNA-binding domains). The *AtMYB26* is involved in lignin and cellulose deposition of endothecium cell walls of anther (Steiner-Lange *et al.* 2003). As *GhMYB9* was dominantly expressed in ovaries and stamens, we speculated that *GhMYB9* may regulate ovary and stamens development by regulating the deposition of lignin and cellulose using a similar function to *AtMYB26*.

The study of fiber development verified that plant hormones including auxin, brassinosteroid, gibberellic acid (GA_3), abscisic acid, and ethylene are the key regulating factor of fiber development (Singh *et al.* 2009). Auxin mainly promotes the elongation of fibers and the thickening of a secondary wall (Liao *et al.* 2010). Through GUS activity staining in the *pF1-GUS* transgenic plants, we found *GhMYB9* predominantly expressed in the fiber elongation period; and the expression was induced by IAA, the result similar to that of Hsu *et al.* (2005). Fibers of the *pF1-GUS* (-1 231 to +122), *pF2-GUS* (-860 to +122), and *pF3-GUS* (-524 to +122) transgenic plants were induced by IAA only in *pF1-GUS*, which demonstrates that the -1 231 to -860 nt region might be essential for gene response to auxin. Although any known auxin response motif was not predicted in the *GhMYB9* promoter sequence, we believe that in the -1 231 to -860 nt region may exist an unknown auxin response motif.

Here, we reported on the R2R3-MYB transcription factor, *GhMYB9*. Through promoter analysis, we found that *GhMYB9* was expressed in the elongation period of cotton fibers, stamens, and ovaries. In addition, we identified that the region from -1 231 to -860 bp in the *GhMYB9* promoter sequence was crucial for auxin response.

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