Control of Plant Trichome Development by a Cotton Fiber MYB Gene [W]

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Cotton (*Gossypium* spp) plants produce seed trichomes (cotton fibers) that are an important commodity worldwide; however, genes controlling cotton fiber development have not been characterized. In *Arabidopsis thaliana* the MYB gene *GLABRA1* (*GL1*) is a central regulator of trichome development. Here, we show that promoter of a cotton fiber gene, *RD22-like1* (*RDL1*), contains a homeodomain binding L1 box and a MYB binding motif that confer trichome-specific expression in Arabidopsis. A cotton MYB protein GaMYB2/Fiber Factor 1 transactivated the *RDL1* promoter both in yeast and in planta. Real-time PCR and in situ analysis showed that *GaMYB2* is predominantly expressed early in developing cotton fibers. After transferring into Arabidopsis, *GL1::GaMYB2* rescued trichome formation of a *gl1* mutant, and interestingly, *35S::GaMYB2* induced seed-trichome production. We further demonstrate that the first intron of both *GL1* and *GaMYB2* plays a role in patterning trichomes: it acts as an enhancer in trichome and a repressor in nontrichome cells, generating a trichome-specific pattern of MYB gene expression. Disruption of a MYB motif conserved in intron 1 of *GL1*, *WEREWOLF*, and *GaMYB2* genes affected trichome production. These results suggest that cotton and Arabidopsis use similar transcription factors for regulating trichomes and that *GaMYB2* may be a key regulator of cotton fiber development.

INTRODUCTION

Aerial surfaces of most land plants have epidermal hairs (trichomes), and some plants of systematically unrelated groups, such as cotton (*Gossypium* spp) and Salix, produce seed trichomes, which are among the longest plant cells. Trichomes are believed to protect plants against insects, microbes, herbivores, and abiotic damages and to assist seed dispersal. Furthermore, seed trichomes of Gossypium plants, namely cotton fibers, are the most widely used natural fibers in the textile industry (Wilkins et al., 2000; Larkin et al., 2003).

Trichomes provide an excellent system for investigation of cell fate determination. In the epidermis, only some cells are committed to becoming a trichome, and the location and timing of trichome differentiation present an example of cell patterning. Once initiated, trichomes often undergo endoduplication and rapid elongation. In addition, trichome production can be genetically manipulated; alterations and even depletion of trichomes often do not affect plant viability and are readily observable (Larkin et al., 2003).

For the model plant *Arabidopsis thaliana*, 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 (Schneider et al., 1997; Schnittger et al.,

1999; Larkin et al., 2003). Two transcription factors, GLABRA1 (GL1) and GL3, function as positive regulators of trichome development, and they encode an R2-R3 MYB protein and a basic helix-loop-helix–related protein, respectively (Oppenheimer et al., 1991; Payne et al., 2000). TRANSPARENT TESTA GLABRA1 (TTG1), a WD40 domain–containing protein that is thought to mediate protein–protein interactions, also acts as a positive regulator (Walker et al., 1999). TRIPTYCHON, CAPRICE, and ENHANCER OF TRY AND CPC have been shown to negatively regulate trichome formation. All three genes encode single-repeat MYB proteins lacking a transcriptional activation domain, and they act in a partially redundant manner to mediate lateral inhibition (Wada et al., 1997, 2002; Schnittger et al., 1999; Schellmann et al., 2002; Kirik et al., 2004). It is proposed that the combinatorial action of these patterning genes regulates trichome-specific expression of *GL2*, which encodes a homeobox (HOX) transcription factor that promotes trichome cell differentiation and growth (Rerie et al., 1994). Thus, GL2 may be considered a regulator of downstream genes of the trichome pathway. However, it has been unclear up to now how these upstream patterning genes, such as *GL1*, are regulated and whether this regulation also involves lateral inhibition.

Although both are unicellular epidermal hairs, cotton fibers are distinct from Arabidopsis trichomes in that they are produced in a specific organ (seed), their initiation and development are highly synchronous, and they are unbranched and extremely elongated (Kim and Triplett, 2001). Analyses of gene expression patterns have identified numerous genes whose transcripts are enriched in fiber cells (John and Crow, 1992; Li et al., 2002; Ji et al., 2003), including those encoding metabolism enzymes that are essential to cell growth (Pear et al., 1996; Ruan et al., 2003). For transcription factors, a noteworthy work is the isolation of six MYB genes from *G. hirsutum* (Loguerico et al.,

1999; Cedroni et al., 2003). Another MYB gene (*GhMYB109*) was recently reported and it is mainly expressed in developing fibers (Suo et al., 2003). Functions of all these cotton MYBs, however, remain for investigation.

In this report, we show that the promoter of a cotton fiber gene, *RD22-like1* (*RDL1*), conducts trichome-specific expression in Arabidopsis plants. By dissecting the promoter, we identified two cotton transcription factors, GaMYB2/Fiber Factor 1 (FIF1) and GhHOX3, which are able to activate the *RDL1* promoter. *GaMYB2* encodes a GL1-like MYB protein and is expressed early in developing fiber cells. In Arabidopsis, *GL1::GaMYB2* fully rescued trichome formation of a *gl1* mutant. We found that regulation of trichome formation by *GL1*-like MYB genes requires an intron (intron 1), which contains a conserved MYB binding site. Interestingly, when expressed under the control of the *35S* promoter, the cotton fiber protein GaMYB2 induced Arabidopsis to produce seed trichomes.

RESULTS

Expression Pattern of Cotton *RDL1*

Our previous study showed that transcripts of a cotton *RDL* gene were enriched in developing fiber cells (Li et al., 2002). DNA gel blot hybridization revealed two *RDL* genes, namely *RDL1* and *RDL2*, in the genome of *G. arboreum* (see Supplemental Figure 1 online), an A-genome diploid cotton cultivar (Rong et al., 2004). RT-PCR analysis showed that whereas *RDL2* was expressed in various cotton tissues except roots (data not shown), *RDL1* was expressed mainly in developing fiber cells (Figure 1A). In ovules, the *RDL1* transcripts were undetectable at the day postanthesis (DPA), abundant in rapidly elongating fibers (3 to 9 DPA), and dramatically decreased after 12 DPA. When fibers were separated from the ovule, *RDL1* transcripts were mostly present in the fiber portion (Figure 1A). These results indicate that *RDL1* is largely a fiber-specific gene.

To investigate the regulatory mechanism of cotton fiber gene expression, a 7-kb upstream segment of *G. arboreum RDL1* gene was isolated by genome walking. According to rapid amplification of cDNA ends (5′-RACE), *RDL1* gene transcription starts at the A within the sequence of CCATTCC, which is located at 77 bp upstream to the translation initiation codon ATG (data not shown). A promoter fragment of ∼1200 bp (*RDL1-P1*) was then fused with a β-glucuronidase (*GUS*) reporter gene. After transferring into Arabidopsis, GUS activity was present exclusively in trichomes (Figure 1B). Clearly, in Arabidopsis the cotton *RDL1-P1* is a trichome-specific promoter.

cis-Elements Conducting Trichome Expression

Successive deletions of *RDL1-P1* from the 5′-end revealed a 302-bp fragment (*RDL1-P3*) proximal to the coding region that was already sufficient to conduct trichome-specific expression. Further deletion showed that two DNA segments (−221 ∼ −77 and −32 ∼ +80 bp) contained all the sequence information necessary for the promoter activity (Figure 2A).

To identify *cis*-acting elements in *RDL1-P3*, 20 DNA replacements were made by PCR-directed linker scanning. We found

that changing of a region by three replacements (SCAN-7, -8, and -9) strongly reduced *RDL1-P3::GUS* expression (Figure 2B). Sequence analysis by PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/) showed that this region contains an L1 box (TGCATTTA) and a MYB motif (CAGTTG), which are separated by 13 nucleotides only (Figure 2B). The L1 box is the binding site of HOX proteins, including the L1 layer-specific ATML1 (Abe et al., 2001) and the trichome regulator GL2 (Ohashi et al., 2003). The MYB motif is recognized by MYB transcription factors; Arabidopsis MYB2, for example, binds to the MYB motif of *RD22* promoter (Urao et al., 1993). The Arabidopsis GL1, which controls trichome formation, and WEREWOLF (WER), which controls development of non-hair cells of root, are both MYB transcription factors (Oppenheimer et al., 1991; Lee and Schiefelbein, 1999).

To see if these two boxes were responsible for trichome expression, we performed site-directed mutations to change the L1 box from TGCATTTA to TGCcgTTA and the MYB motif from CAGTTG to CAGTgG. Either mutation reduced the promoter activity greatly, though weak GUS staining in trichomes was still observable. Mutation of both *cis*-elements, however, completely inactivated the promoter (Figure 2C). These results indicate that both L1 box and MYB motif are involved in activating the *RDL1-P3* promoter in Arabidopsis trichomes.

Transcription Factors Binding to *RDL1-P3*

Because both L1 box and MYB motif contribute to the trichome expression of *RDL1-P3::GUS*, it is possible that the trichome factors of GL1 and GL2 transactivated *RDL1-P3* in transgenic Arabidopsis, and their cotton homologs regulate *RDL1* expression in cotton fiber cells.

Searching the database revealed three cotton MYBs that might be related to trichome development. GhMYB1 (Loguerico et al., 1999), also called CotMYBA (Payne et al., 1999), is 33% identical to *Antirrhinum majus* MIXTA, a positive regulator of floral papillae development (Noda et al., 1994). GhMYB2 (Loguerico et al., 1999) and GhMYB109 (Suo et al., 2003) are 47% identical to each other, and they show 44 and 46% sequence identities to GL1, respectively (see Supplemental Figure 2 online). To avoid confusion with Arabidopsis MYB2, which is not related to trichome development, the cotton MYB2 was also named FIF1 during our investigation. For homeobox proteins, GhHOX1 (AF5309130) and GhHOX2 (AF530914) show 66 and 34% sequence identities to GL2, respectively. We then isolated all these genes or cDNAs from *G. arboreum*, in addition to a cDNA encoding a novel HOX protein, GhHOX3, which is 37% identical to GL2. These cotton transcription factors were then investigated further.

In yeast one-hybrid system, the *G. arboreum* MYB2 and MYB109 as well as Arabidopsis GL1 and WER exhibited a clear binding activity to a *RDL1* promoter fragment (−221 ∼ −33) that contained both the L1 box and MYB motif, whereas another cotton MYB protein, GaMYB1, did not, though its MYB motif showed 65% sequence identity to that of GaMYB2. As mentioned above, GaMYB1 is more similar to Antirrhinum MIXTA than to GaMYB2 and Arabidopsis GL1. When the MYB motif was mutated from CAGTTG to CAGTgG, the MYB proteins were no longer able to bind the DNA (Figure 3A). All the cotton and
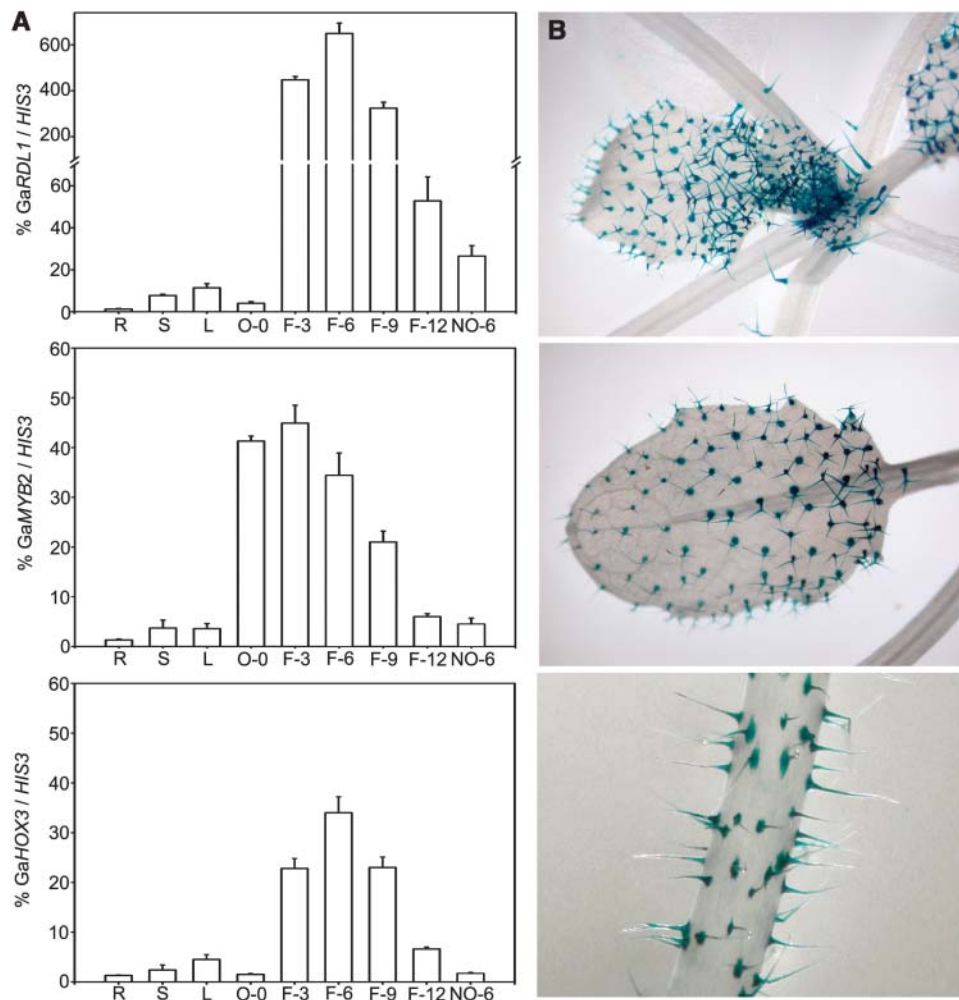
Figure 1. Expression Patterns of *G. arboreum RDL1*, *MYB2/FIF1*, and *HOX3* Genes.

(A) Transcript profile of *GaRDL1*, *GaMYB2*, and *GaHOX3* in plants of *G. arboreum*. Relative transcript amounts of each gene were normalized with respect to cotton *Histone3* transcript levels (100%). Mean values were obtained from three independent PCR amplifications, and the error bars indicate the standard error of the mean. A break in the scale (=) has been incorporated to show the higher amount of *GaRDL1* in fibers. R, roots; S, stems; L, leaves; O-0, 0-DPA ovules; F-3, 3-DPA fibers; F-6, 6-DPA fibers; F-9, 9-DPA fibers; F-12, 12-DPA fibers; NO-6, 6-DPA naked ovules (fibers stripped off).
(B) GUS staining of Arabidopsis plants expressing *RDL1-P3*::*GUS*. Young leaf (top), mature leaf (middle), and stem (bottom) are shown.

Arabidopsis HOX proteins tested, however, did not bind to this fragment in yeast, probably because the HOX proteins fused with GAL4 activation domain were too large to be properly expressed or the binding would require other plant proteins to form a complex.

To provide in planta evidence of *RDL1-P3* regulation, related MYB and HOX genes were expressed in wild-type plants of Arabidopsis under the control of the *35S* promoter, and their effects on *RDL1-P3*::*GUS* expression were examined. We found that ectopic expression of the MYBs, including *GaMYB2*, *GL1*, and *WER*, induced a weak expression of *RDL1-P3*::*GUS* in nontrichome cells but repressed its expression in trichomes. In plants expressing *35S*::*GL2* or *35S*::*GhHOX3*, the GUS activity was clearly present in trichomes, in addition to a relatively low activity in nontrichome cells. When the MYB and the HOX genes

(*GL1* and *GL2* or *GaMYB2* and *GaHOX3*, respectively) driven by the *35S* promoter were cotransferred into Arabidopsis, a strong GUS staining was observed in both trichome and nontrichome cells (Figure 3B), resembling the ubiquitous pattern of the *35S* promoter. For each pair of transcription factors, the effects seemed to be synergistic rather than additive (Figure 3C). These results suggest that GL1-like MYB and GL2-like HOX proteins operate together to activate the *RDL1-P3* promoter in Arabidopsis plants. In addition, we also transformed the Arabidopsis plants with *35S*::*GaMYB2*/*35S*::*HOX1* and *35S*::*GaMYB2*/*35S*::*HOX2*. We found that, unlike *35S*::*GaMYB2*/*35S*::*HOX3*, these two combinations did not activate the *RDL1* promoter efficiently (data not shown), suggesting that there might be a specific cooperation between GaMYB2 and HOX3 in regulating the *RDL1* promoter.
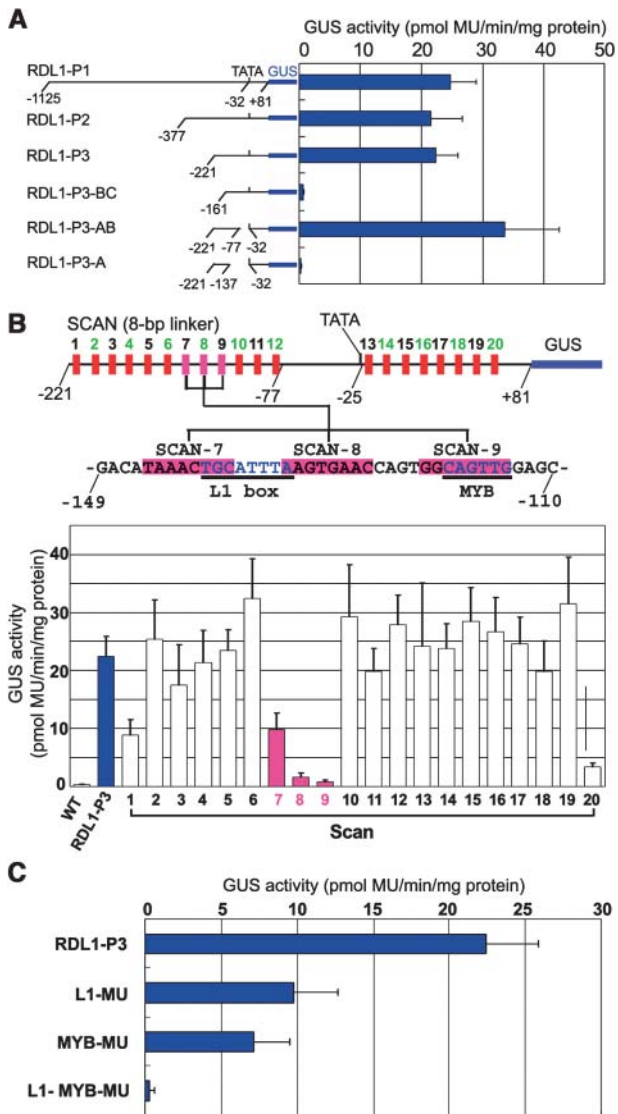
A

B

C

Figure 2. Identification of *cis*-Elements in the *RDL1* Promoter.

(A) GUS activities driven by different upstream fragments of the cotton *RDL1* gene. Nucleotides are numbered from the transcription initiation site.

(B) Linker scanning of *RDL1-P3*. The 8-bp linkers (GGAATTCC or CCTGCAGG) were used. Red and pink boxes represent linkers of SCAN-1 through -20. The L1 box and MYB motif are underlined.

(C) Effects of disruption of L1 box (L1-MU) and MYB motif (MYB-MU) on promoter activity. L1-MYB-MU indicates that both L1 box and MYB motif were mutated. The L1 box was mutated from TGCATTTA to TGCcgTTA and the MYB motif from CAGTTG to CAGTgG.

Complementation of Cotton *MYB2/FIF1* to *gl1* Mutant

To see if GaMYB2 is functionally similar to GL1, complementation was performed on an Arabidopsis *gl1* mutant (SALK_039478), which has a T-DNA inserted in the third exon, resulting in a glabrous phenotype (Figure 4A). It is thus a loss-of-function mutation. The coding region of *GaMYB2* gene was then

placed between the upstream and downstream segments of the *GL1* gene (*GL1::GaMYB2*); after transferring into *gl1* plants, the transgene fully rescued trichome production in >90% of the transformants, similar to the efficiency obtained with *Gl1::GL1* and *GL1::WER* (Figure 4B, Table 1). When the three MYB genes driven by the *35S* promoter were used for complementation, normal trichome production was also observed in plants of T1 generation. In the wild-type background, these MYB genes driven by either the *GL1* sequences or the *35S* promoter caused reduced production of leaf trichomes in 30 ∼ 50% of the transgenic plants (Figure 4B, Table 1). Previous studies also showed two opposite effects of *35S::GL1* expression: suppressing trichome production in the wild-type background and promoting trichome formation in *gl1* (Oppenheimer et al., 1991; Larkin et al., 1994), but such effect has not been reported before for MYB genes driven by the *GL1* regulatory sequences. Reciprocal complementation using *GL1* and *WER* found that the two genes encode functionally equivalent MYB proteins, though they specify distinct cell types because of nonoverlapping expression patterns (Lee and Schiefelbein, 2001). Our complementation data presented here indicate that the cotton protein GaMYB2 has the same function as Arabidopsis GL1 and WER in regulating trichome development.

Intron 1 Is Required for Patterning Gene Expression

The Arabidopsis *GL1* and *WER* genes contain two introns, whereas the cotton genes of *GaMYB2* and *MYB109* contain only one. The cotton intron is equivalent to the first intron of Arabidopsis genes regarding their positions (131 to 146 bp downstream of the translation start codon ATG). During our complementation tests, we also used *GaMYB2* cDNA (*GaMYB2c*). Surprisingly, neither *GL1::GaMYB2c* nor *35S::GaMYB2c* rescued trichome formation of the *gl1* mutant. In the wild-type background, the two constructs did not exhibit any effect on trichome production. We then extended this test to Arabidopsis *GL1* and *WER*. Under the control of either *GL1* sequences or the *35S* promoter, the intronless cDNAs did not work (Figure 4B, Table 1). This intron requirement is therefore common to these trichome-related MYB genes, rather than specific to *GaMYB2*.

When the trichome-specific promoter *RDL1-P3* was used to drive *GaMYB2*, both the intron 1–containing genomic coding region and the cDNA functioned in regulating trichome development in plants of T1 generation (Figure 4C). Similar results were obtained with *GL1* (Table 1). In a previous study, *ATML1::GL1c* also rescued the *gl1* mutant defects (Esch et al., 2003). The *ATML1* promoter is expressed at a low level in all the epidermal cells of the developing leaf, and in situ analyses have shown its enhanced expression in young developing trichomes (Sessions et al., 1999). Therefore, cDNAs of the MYB genes could work normally, at least in T1, if expressed properly. These results imply that the intron is involved in regulating the gene expression pattern.

To confirm this assumption, we constructed two types of chimerical *GL1::GUS* reporter genes containing different introns in the coding region: one contained the *GUS* intron in pCAMBIA1301, and the other employed instead the *GaMYB2*
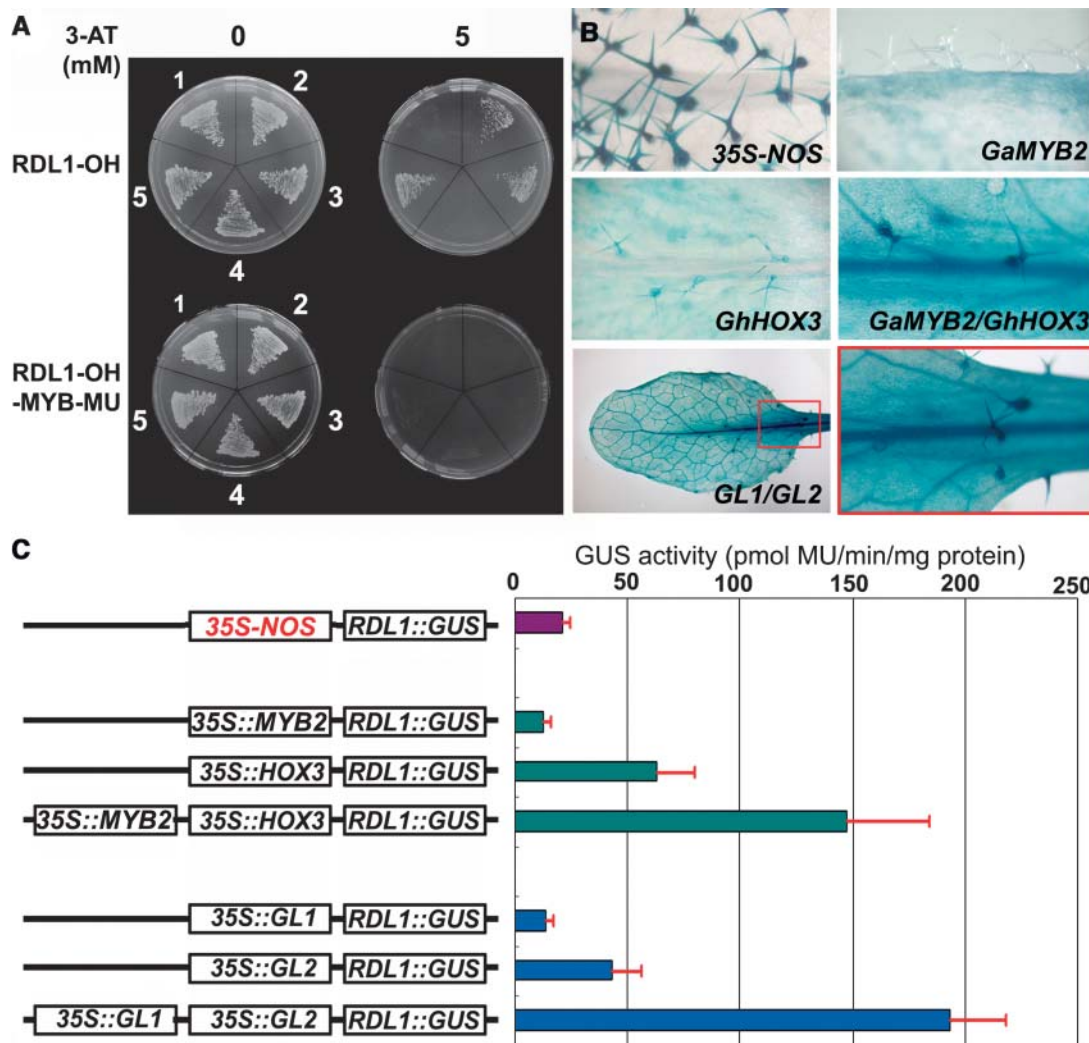
Figure 3. Transcriptional Activation of *RDL1-P3* by GaMYB2 and HOX3.

(A) DNA–protein interactions in yeast one-hybrid system. Yeast strain YM4271 integrating *RDL1-OH* (−221 to −33 bp) or *RDL-OH-MYB-MU* was transformed with GAL4 transcriptional activation domain (AD) (1), AD-GL1 (2), AD-WER (3), AD-GaMYB1 (4), and AD-GaMYB2 (5).
(B) Effects of ectopic expression of MYB (*GaMYB2* or *GL1*) and HOX (*HOX3* or *GL2*) genes on the *RDL1-P3::GUS* expression pattern in Arabidopsis. In *GL1/GL2*, the box indicates the magnified region shown at right. *35S-NOS*, the *35S* promoter and nopaline synthase (*NOS*) terminator cassette; *GaMYB2*, *35S::GaMYB2/RDL1::GUS*; *HOX3*, *35S::HOX3/RDL1::GUS*; *GaMYB2/HOX3*, *35S::GaMYB2/35S::HOX3/RDL1::GUS*; *GL1/GL2*, *35S::GL1/ 35S::GL2/RDL1::GUS*.
(C) GUS activities in Arabidopsis plants expressing both *RDL1-P3::GUS* and the transcription factors as indicated. *MYB2*, *GaMYB2*.

intron or *GL1* intron 1. We found that, when the MYB intron was present, GUS activity was restricted to trichomes and the signal was clear. In plants expressing the original *GUS* gene, the GUS staining pattern was far from specific (Figure 5A).

Identification of an Intronic MYB Motif

PLACE analysis identified a MYB binding motif (CA/CGTTA) that is present in the *GaMYB2* intron and in the first intron of *GL1* and *WER* as well. Furthermore, position of this motif is conserved (22 or 23 bp upstream of the second exon) (Figure 5B). This intronic

MYB motif is not found in *MYB109* of *G. arboreum*. We then mutated this MYB motif into CA/CacTA. We found that the activity of promoting trichome formation in *gl1* plants was considerably weakened after mutation, although the degree was different depending on the individual genes (Figure 5C, Table 1). For *GL1::GL1-IN-MU*, though the complementation rate was still high, >40% of transgenic plants showed abnormal trichome production: the amount of leaf trichomes was decreased and trichomes were mainly distributed on the leaf margin and stem. For *GL1::GaMYB2-IN-MU*, this mutation cost >50% of the transgenic plants without complementation. And for
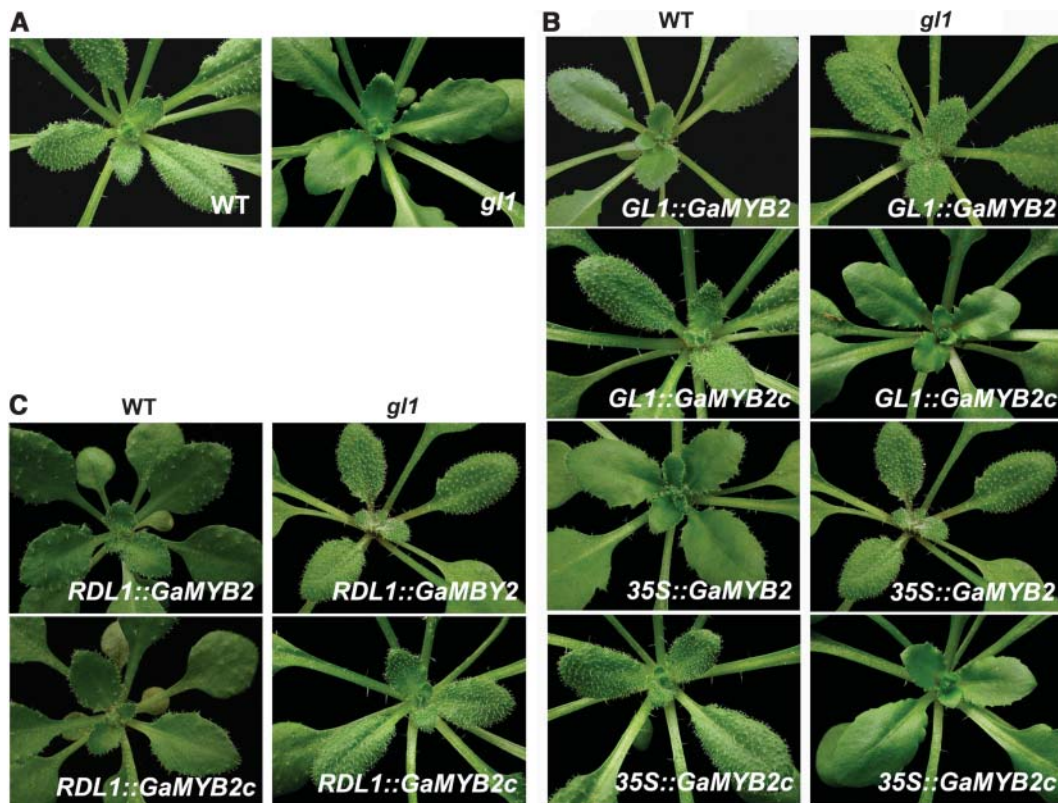
Figure 4. GaMYB2 Regulates Arabidopsis Trichome Development.

(A) The Arabidopsis wild-type and a *gl1* (SALK_039478) mutant seedling.

(B) and **(C)** Trichome phenotypes of wild-type or *gl1* mutant plants transformed with various chimerical genes as indicated. For intronless cDNA, a ''c'' was added as a suffix.

GL1::*WER-IN-MU*, most of the trichome-regulating activity was lost. When *GL1*::*GaMYB109* was introduced into *gl1* plants, only a partial complementation was achieved (Figure 5C, Table 1), consistent with the fact that the intronic MYB motif is not present. These results indicate that this intronic MYB motif of *GL1*-related MYB genes plays a role in regulating trichome production.

Expression Patterns of *GaMYB2*

In Arabidopsis, the cotton *GaMYB2* gene showed a high activity of controlling trichome formation. If this MYB factor, which is encoded by a single gene in *G. arboreum* (see Supplemental Figure 1 online), controls cotton fiber development, it should have a higher level of expression in fiber cells at an early stage of ovule development. Real-time PCR showed that *GaMYB2* transcripts were indeed highly enriched in developing fiber cells, and the expression level was either rather low or undetectable in all other tissues examined (Figure 1A). A similar expression pattern was observed for the *HOX3* and *RDL1* genes; however, at the very early stage of ovule development (0 DAP), only *GaMYB2* exhibited a substantial level of expression (Figure 1A). In situ mRNA hybridization of 0-DPA ovules localized the *GaMYB2* transcripts in fiber initials (Figure 6A), confirming its fiber-specific expression in ovules.

Promoting Arabidopsis Seed-Trichome Production by *GaMYB2*

Seeds of Arabidopsis are completely glabrous. Our extensive observation of a large number of samples under binoculars did not find any trichomes on seed coat of wild-type plants. We then examined seeds of transgenic *35S*::*GaMYB2* plants. Interestingly, the transgene promoted seed-trichome formation in both wild-type and *gl1* backgrounds. In the wild-type background, we found that ~20% (11/54) of T1 plants showed seed-trichome phenotype, among these plants ~20% (82/416) of the seeds produced epidermal trichomes. The trichomes were often unbranched or occasionally branched, 0.1 to 0.2 mm long (Figures 6B and 6C), and the number of trichomes on each seed was small: frequently one and at most four. Other aspects of seed development appeared normal. In plants transformed with *35S*::*GL1*, *35S*::*WER*, and *35S*::*GaMYB109*, seed trichomes were not observed.

DISCUSSION

Dissection of the cotton *RDL1* promoter indicated that both L1 box and MYB motif contribute to its activity of trichome-specific expression in Arabidopsis, and both elements confer a positive

Table 1. Regulation of Arabidopsis Trichome Development by *GL1*-Like Genes

Construct	Wild-Type Background			gl1 Background			
	T1 Plants Examined	+ (%)	↓ (%)	T1 Plants Examined	+ (%)	+/− (%)	− (%)
GL1::GaMYB2	47	57.5	42.5	54	92.6	0.0	7.4
GL1::GaMYB2c	46	100.0	0.0	52	0.0	0.0	100.0
GL1::GaMYB109	48	100.0	0.0	51	11.8	64.7	23.5
GL1::GaMYB109c	48	100.0	0.0	50	0.0	0.0	100.0
GL1::GL1	48	56.2	43.8	54	96.3	0.0	3.7
GL1::GL1c	46	100.0	0.0	54	0.0	0.0	100.0
GL1::WER	48	52.1	47.9	53	100.0	0.0	0.0
GL1::WERc	48	100.0	0.0	54	0.0	0.0	100.0
35S::GaMYB2	54	68.5	31.5	54	92.6	0.0	7.4
35S::GaMYB2c	52	100.0	0.0	54	0.0	0.0	100.0
35S::GaMYB109	54	100.0	0.0	48	0.0	0.0	100.0
35S::GaMYB109c	50	100.0	0.0	48	0.0	0.0	100.0
35S::GL1	54	66.7	33.3	53	98.1	0.0	1.9
35S::GL1c	54	100.0	0.0	50	0.0	0.0	100.0
35S::WER	53	66.0	34.0	54	100	0.0	0.0
35S::WERc	53	100.0	0.0	52	0.0	0.0	100.0
RDL1::GaMYB2	32	72.9	28.1	30	96.7	0.0	3.3
RDL1::GaMYB2c	31	74.2	25.8	32	100.0	0.0	0.0
RDL1::GL1	31	77.4	22.6	32	100.0	0.0	0.0
RDL1::GL1c	32	78.1	21.9	31	100.0	0.0	0.0
GL1::GaMYB2-IN-MU	36	69.4	30.6	36	11.1	33.3	55.6
GL1::GL1-IN-MU	36	63.9	36.1	36	58.3	41.7	0.0
GL1::WER-IN-MU	36	61.1	38.9	36	11.1	0.0	88.9

+, Trichomes normal; ↓, trichome amount reduced (less than one-third of wild-type trichomes); +/−, partial complementation to *gl1* (less than half of wild-type trichomes); −, glabrous.

regulation. It is interesting that the 302-bp DNA fragment of *RDL1-P3* is already sufficient to drive trichome-specific expression efficiently. Previously, a 334-bp promoter of a tobacco (*Nicotiana tabacum*) P450 gene (*CYP71D16*) was shown to drive gene expression in distal cells of multicellular trichomes (Wang et al., 2002). These short fragments, particularly the two *cis*-elements identified here, can be used to design artificial promoters for engineering trichome-based molecular farming, as well as fiber trait improvement.

GL1 is the first transcription factor cloned that controls Arabidopsis trichome development (Herman and Marks, 1989; Marks and Feldmann, 1989). The prevalent model is that GL1, GL3, and probably TTG1 form a transcription complex that regulates expression of *GL2*, and this homeobox protein regulates downstream target genes to control epidermal cell differentiation (Szymanski et al., 1998; Ohashi et al., 2003). Besides *GL2*, no other target genes have been identified for GL1, although they were suspected to exist (Larkin et al., 2003). We demonstrate in this paper that GL1-like MYB proteins (GL1, WER, and GaMYB2) are able to bind to the MYB motif of the cotton *RDL1* promoter, and both the MYB and HOX proteins are involved in transcriptional regulation of this promoter. Considering that the L1 box and MYB motif are located close to each other in *RDL1-P3*, the MYB and HOX proteins may form a complex that transactivates this promoter.

This type of trichome-gene regulation is not an isolated case, for the cotton *LTP3* gene, which encodes a putative lipid transfer protein, also has L1 box and MYB motifs in its upstream sequence; in transgenic tobacco plants, the *LTP3* promoter directed trichome-specific expression of *GUS* as well (Liu et al., 2000). In cotton ovules, transcripts of both *RDL1* and *LTP3* genes are distributed mainly in developing fiber cells. It is thus highly possible that the two elements are recruited by cotton transcription factors to confer fiber cell expression of a group of cotton genes. Because both *RDL1* and *LTP3* are cotton genes, we searched the Arabidopsis genome database to find endogenous genes that have a similar promoter. We then identified several candidates, including a putative membrane bound *O*-acyl transferase gene (At5g55330). Promoter of this gene indeed directs trichome-specific expression, and its expression requires GL1 (our unpublished results). Based on these findings, we propose that similar molecular machinery is shared by cotton fibers and Arabidopsis trichomes in regulating a subset of downstream target genes, which involves both the GL1-like MYB and GL2-like HOX transcription factors.

Because function of a cell fate–determining gene is closely related to its expression pattern, regulatory elements are of central importance. Previous investigations of *GL1* expression provided a vague picture. A promoter from the *GL1* 5′ noncoding region directed *GUS* expression in stipules only (Oppenheimer et al., 1991), whereas in situ hybridization showed a predominant distribution of the transcripts in developing trichomes and protoderm cells; the downstream sequence was then suggested to be required for normal expression (Larkin et al., 1993). In this
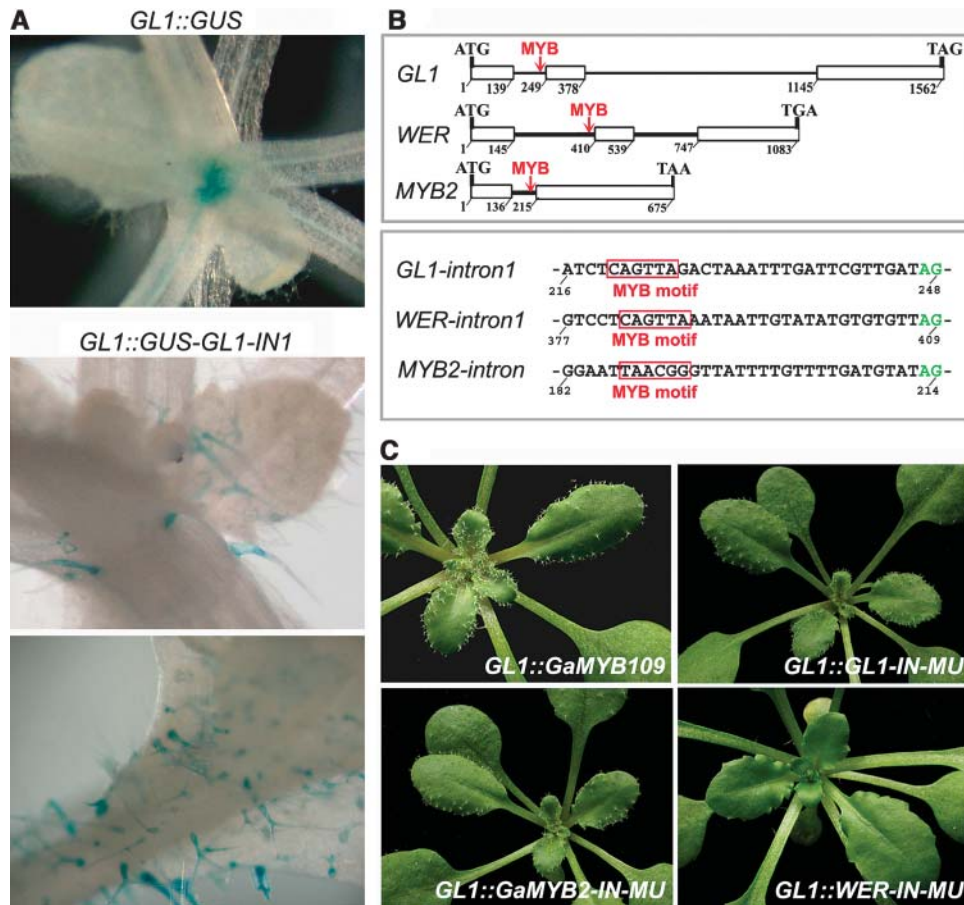
Figure 5. Effects of Intron 1 and the Intronic MYB Motif on Expression of Trichome-Related Genes.

(A) GUS staining of Arabidopsis seedlings expressing *GL1*::*GUS* containing the *GUS* intron (top) and the first intron of *GL1* (middle and bottom).
(B) Intron positions in *GL1*, *WER*, and *GaMYB2* genes and the MYB motif in the first intron of each gene. *MYB2*, *GaMYB2*.
(C) Reduced production of trichomes in *gl1* plants expressing *GL1*::*GaMYB109*, whose intron contains no MYB motif, or the MYB genes with disrupted intronic MYB motif. See also Table 1.

study, we show that the trichome-specific expression can be achieved as long as intron 1 is present. This intron can be considered a repressor in nontrichome cells because it prevented ectopic expression of the MYB genes in nontrichome cells. On the other hand, despite the restricted expression domain because of the presence of this intron, the overall transcript level still increased (data not shown), and the GUS signal in trichomes was comparatively strong (Figure 5A); these data suggest that the intron also acts as an enhancer in trichome cells. This cell-specific bidirectional regulation mediated by the intron identifies with the lateral inhibition model proposed for trichome formation.

That a MYB binding motif is found in the intron (intron 1) of *GL1*, *WER*, and *GaMYB2* is unlikely a coincidence because this motif is required for normal trichome production. However, unlike deletion of the intron, disruption of this motif reduced, rather than eliminated, the trichome-regulating potential (Table 1). This suggests that the intron may contain other *cis*-elements. Alternatively, intron removal by a spliceosome may play an additional role.

35S::*GL1* but not *35S*::*GL1c* rescued the trichome formation of the *gl1* mutant. Elaboration of trichomes by *35S*::*GL1* or related transgenes is likely owing to the intron (intron 1) because the *35S* promoter is active in practically all tissues. However, unlike *GL1*::*GL1*, *35S*::*GL1* could not maintain a normal trichome production in plants of T2 and subsequent generations. This indicates that the 5′ and 3′ noncoding sequences of *GL1* are also involved in patterning trichome formation. Further dissection is required to clarify the regulatory role for each of these DNA segments.

Most eukaryotic genes are interrupted by one or more introns, which can influence many aspects of gene expression. In plants, a well-characterized example is Arabidopsis *AGAMOUS* (*AG*), a MADS box gene required for the development of the internal two whorls (stamens and carpels) of flower. Proper expression of *AG* involves sequences located in the second intron (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000; Lohmann et al., 2001; Hong et al., 2003). Besides intronic *cis*-acting elements, presence of the first intron may enhance plant gene
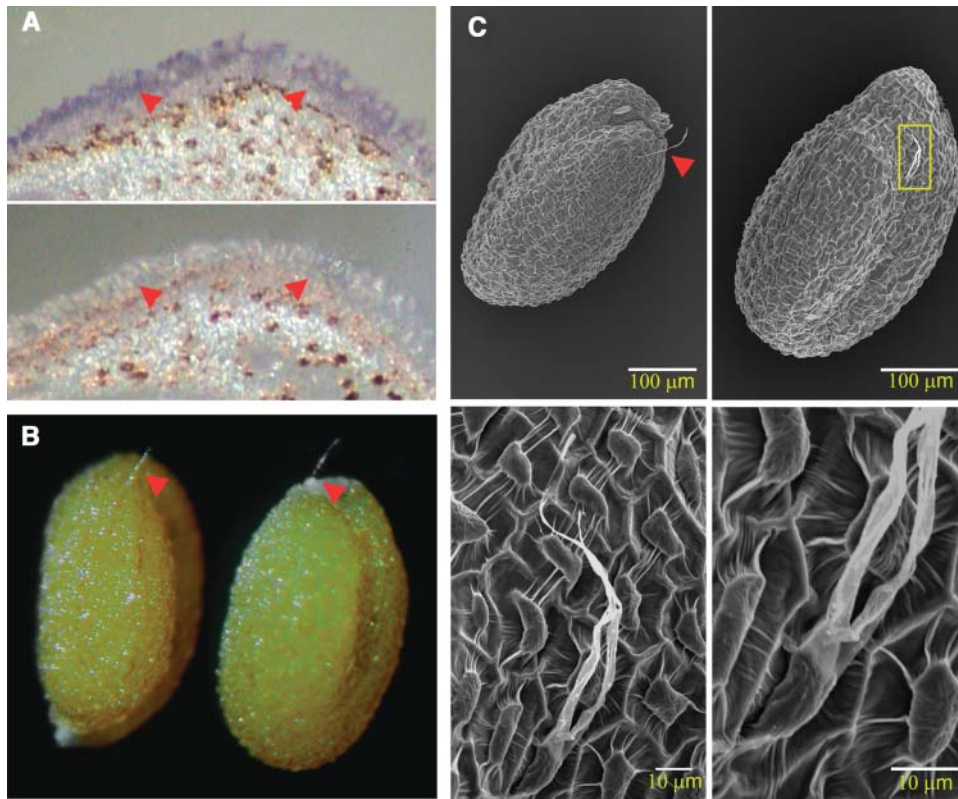
Figure 6. In Situ Hybridization of Cotton Ovule and the Arabidopsis Seed Trichome.

(A) In situ hybridization of *GaMYB2* transcripts in the *G. arboreum* ovule (0 DPA) with antisense (top) and sense (middle) probes. Arrowheads indicate fiber cells; the mRNA was localized in fiber cells.
(B) Trichome(s) on seed of wild-type Arabidopsis expressing *35S*::*GaMYB2* observed under binoculars. Arrowheads indicate the seed-trichome.
(C) Scanning electron micrographs depicting the trichome on seed of transgenic Arabidopsis expressing *35S*::*GaMYB2*. The box indicates the magnified region shown in the bottom panels. Arrowhead indicates the seed-trichome.

expression (Callis et al., 1987; Fu et al., 1995; Cai et al., 1998). It is now clear that proper expression of *GL1* also requires the first intron. This intron-involved regulation of trichome formation is likely conserved in flowering plants because Arabidopsis and cotton represent two distantly related families.

Arabidopsis trichomes and cotton fibers are both unicellular hairs of epidermis, and they may share similar molecular machinery of regulation. Since the cloning of the Arabidopsis *GL1* gene, scientists have been searching for MYB transcription factors that control cotton fiber initiation and development. GhMYB1 shows certain homology to MIXTA; when expressed in tobacco under the *35S* promoter, it caused phenotypic changes that were global rather than specific, though supernumerary trichomes were observed. In Arabidopsis, however, its expression did not alter trichome production (Payne et al., 1999); and in our yeast one-hybrid assay, GaMYB1 failed to bind to the *RDL1* promoter (Figure 3A). The cotton MYB109 shows a relatively high homology to GL1. However, *GL1*::*MYB109* complemented *gl1* mutation of Arabidopsis only partially (Table 1), and its transcripts were barely detectable in ovules at 0 DPA (Suo et al., 2003), when fiber initiation already started (Kim and Triplett, 2001). It is unlikely involved in early initiation of cotton fibers.

Three lines of evidence presented here suggest the cotton MYB2 to be a key regulator of cotton fiber development. First, it showed a clear binding activity to the cotton fiber-specific promoter *RDL1* in yeast and, together with cotton HOX3, it regulated *RDL1*::*GUS* expression in Arabidopsis plants. Second, it is highly expressed in fiber cells at an early developmental stage, preceding the expression of other fiber genes, such as *RDL1*, *HOX3* (Figure 1A), and *MYB109* (Suo et al., 2003). Clearly, the spatial and temporal expression pattern of *GaMYB2* fits its proposed role of controlling fiber cell formation. Third, GaMYB2 protein not only rescued trichome formation of an Arabidopsis *gl1* mutant but also promoted the model plant to produce seed trichomes. Besides influencing trichome formation, GaMYB2 did not exhibit other observable effects on Arabidopsis development. Currently, we are transferring sense and antisense constructs of *GaMYB2* into cotton plants to obtain more direct evidence of its role in regulating cotton fiber development.

It has been indicated that similar MYBs may play distinct roles depending on cell types in which they are expressed (Glover et al., 1998; Lee and Schiefelbein, 2001). It is therefore not totally unexpected that *35S*::*GaMYB2* triggered seed-trichome production in Arabidopsis because seed coat should be included in

the expression domain of the *35S* promoter. Previous investigations reported that Arabidopsis plants expressing *35S*::*GL1* produced one to several ectopic trichomes on the cotyledon, but trichomes on seed were not mentioned (Larkin et al., 1994). In this investigation, we did not find any seed trichomes in plants expressing either *35S*::*GL1* or *35S*::*WER*. Thus, the possibility exists that a specific structure of cotton MYB2 is related to its activity of promoting seed–trichome production. Although leaf trichomes are commonly found in flowering plants, seed-trichome formation is a sporadic event of evolution that happened in several unrelated plant families, including Malvaceae, Salicaceae, and Bombacaceae. Dissection of functional domains of cotton MYB2 and related MYB proteins holds a potential of probing into the mystery of seed-trichome evolution.

METHODS

Plant Material

Cotton plants (*Gossypium arboreum* cv Qingyangxiaozi and *G. hirsutum* cv Xuzhou-142) were grown in a greenhouse. Ovules were harvested at various developing stages; fibers were collected by scraping the ovule in liquid nitrogen. Plants of *Arabidopsis thaliana* (Columbia-0) were grown indoors at 22°C under continuous illumination. A T-DNA insertion mutant of *gl1* (SALK_039478) was obtained from ABRC (Ohio State University, Columbus, OH). Arabidopsis tissues above the hypocotyl of the 2-week-old seedlings (four pairs of visible leaves) were collected for analysis, unless otherwise indicated.

DNA and RNA Analysis

Genomic DNA and total RNA were extracted as described (Zhu et al., 2003). For DNA gel blot analysis, hybridization and washing were performed as described (Sambrook et al., 2001). Probes were generated using the Prime-a-Gene Labeling System (Promega, Madison, WI).

Genomic walking was performed on *G. arboreum* DNA to isolate *RDL1* 5′ upstream segment according to the Genome Walker kits (Clontech, Palo Alto, CA). To obtain the 5′-terminal sequence of *G. arboreum RDL1* transcript, 5′-RACE was performed using the SMART RACE cDNA amplification kit (Clontech).

The cDNAs used in this investigation were amplified by RT-PCR from the total RNAs, which were isolated from *G. arboreum* ovules and fiber cells, and from *A. thaliana* seedlings, respectively. *GhHOX3* was isolated from a *G. hirsutum* cDNA library (Zhu et al., 2003). The genes or gene fragments were PCR amplified from the genomic DNA. Pyrobest DNA polymerase (TaKaRa, Dalian, China) was used for the PCR amplification.

The mRNA was quantitatively analyzed by real-time PCR, with gene-specific TagMan primers designed using Primer Express (Applied Biosystems, Foster City, CA), and the primers were given in Supplemental Table 1 online. The 5′ and 3′ ends of these probes were labeled with fluorescent dyes FAM (6-carboxyfluorescein) and TAMRA (6-carboxy-tetramethyl-rhodamine), respectively. Total RNA of 1 μg from various tissues of *G. arboretum* was reverse transcribed in a 20-μL reaction using the RNA PCR (AMV) kit (TaKaRa). TaqMan RT-PCR assays were performed on a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia). In a final volume of 25 μL, the reaction contained 25 ng of reverse transcription mixture, 0.2 μM forward and reverse primers, and 0.1 μM probe. All reactions were performed in triplicate. Cotton *Histone3* (AF024716) mRNA was used as an internal control, and the relative amounts of the mRNA were calculated using the comparative threshold cycle method. Amplification efficiency (82 to 90%) for the four primer-probe sets was determined by amplification of cDNA reverse transcribed from a dilution series of total RNA using 1, 0.5, 0.25, and 0.125 μg per reaction (data not shown).

DNA Mutations and Linker Scanning

Deletion derivatives were PCR constructed using sets of primers (see Supplemental Table 2 online). Linker scanning and point mutations were performed as described (Gustin and Burk, 2000). After PCR amplification, the DNA fragments were inserted into the *Hin*dIII-*Bam*HI sites of pBI121 vector (Clontech) to replace the *35S* promoter region. Presence of the expected mutations was confirmed by sequencing.

Yeast One-Hybrid Assay

Yeast one-hybrid assay was performed using the MATCHMAKER one-hybrid system (Clontech). An *RDL1* promoter region (−221 to −33 bp), RDL1-OH, was ligated into the *Eco*RI-*Xba*I sites of pHISi-1 and *Hin*dIII-*Sal*I sites of pLacZi, respectively. These two bait constructs were linearized with *Xho*I and *Nco*I, respectively, and integrated into the yeast genome (strain YM4271). The level of background HIS was determined by culturing the yeast strain on the synthetic dropout (SD)/-His medium containing various concentrations of 3-amino-1, 2, 4-triazole. A yeast dual reporter strain was prepared by first integrating pLacZi-RDL1, followed by pHISi-1-RDL1, into the genome of yeast. Similarly, yeast strain YM4271 integrating RDL1-OH-MYB-MU was formed and tested. The dual reporter strain was selected and maintained on SD/-His/-Ura medium. For construction of the GAD-MYB or GAD-HOX fusions, the cDNA (open reading frame) was ligated with GAL4 activation domain in pGAD424. Yeast transformants were tested on SD/-Leu/-His/-Ura medium containing 5 mM 3-amino-1, 2, 4-triazole.

Constructs

RDL1-P3, a 302-bp fragment of *RDL1* promoter, was used to substitute the *35S* promoter region in the *Hin*dIII-*Nco*I sites of pCAMBIA1301 (CAMBIA, Canberra, Australia), forming pRDL1::GUS. The *35S* promoter and *NOS* terminator were isolated from pBI121 by PCR and inserted into the *Eco*RI-*Sac*I and the *Sph*I-*Hin*dIII sites of pRDL1::GUS, respectively. Coding regions of MYB and HOX genes were isolated by PCR with gene-specific primers (see Supplemental Table 2 online) and placed between the *35S* promoter and *NOS* terminator, generating p35S::MYB/ RDL1::GUS and p35S::HOX/RDL1::GUS, respectively. The 35S-GL1-NOS cassette was inserted into the *Eco*RI site of p35S::GL2/RDL1::GUS to generate p35S::GL1/35S::GL2/RDL1::GUS. Similarly, the construct of p35S::GaMYB2/35S::HOX3/RDL1::GUS was formed.

The *NOS* terminator was inserted into the *Hin*dIII and *Sph*I sites of pCAMBIA1301, forming p1301-NOS. A 1400-bp upstream fragment and an 1838-bp downstream fragment of Arabidopsis *GL1* gene were amplified by PCR. After sequencing, they were inserted into the *Sph*I-*Pst*I and *Sal*I-*Xba*I sites of p1301-NOS, respectively, forming pGL1-1. Coding regions of the MYB genes and their cDNAs were inserted into the *Pst*I-*Sal*I sites between the 5′-upstream and 3′-downstream fragments of *GL1*, generating pGL1::GaMYB2, pGL1::GaMYB2c, and related constructs of other MYB genes.

RDL1-P3 was inserted into the *Hin*dIII-*Pst*I sites of pCAMBIA1301 to generate p1301-RDL1. Then, *NOS* terminator was inserted into the *Hin*dIII and *Sac*I-*Eco*RI sites of p1301-RDL1 to generate p1301-RDL1-NOS. The cDNAs or genomic coding regions of MYB genes were inserted into the *Pst*I-*Sal*I sites between the *RDL1-P3* and *NOS* of p1301-RDL1-NOS, generating pRDL1::*GaMYB2* and related constructs.

The pGL1-1 was digested with *Bst*EII to remove *GUS*, and part of *GL1* upstream fragment was also removed because it contained a *Bst*EII site.

This *GL1* upstream fragment was then obtained by PCR and inserted back into *Bst*EII, forming pGL1-2. The coding region of the *GUS* gene was isolated from pCAMBIA1301 by PCR and inserted into the *Pst*1 and *Sal*I sites of pGL1-2. The intron of *GUS* gene was replaced by *GL1* intron 1 or *GaMYB2* intron, and modified *GUS* coding regions were inserted into the *Pst*1 and *Sal*I sites of pGL1-2, generating pGL1::GUS-GL1-IN1 and pGL1::GUS-GaMYB2-IN, respectively.

Plant Transformation and GUS Assay

Binary constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and subsequently transferred into Arabidopsis using a floral dip method (Clough and Bent, 1998). Transgenic plants were selected on media containing 50 µg/mL of kanamycin or hygromycin.

Histochemical and quantitative GUS assays were performed as described (Jefferson et al., 1987). Samples were stored in 70% ethanol before microscopic observation. For quantitative assays, fluorescence was measured in a minifluorometer (Hofer, San Francisco, CA).

In Situ Hybridization and Scanning Electron Microscopy

G. arboreum ovules were collected at 0 DPA and embedded in paraffin. *GaMYB2* mRNA was detected with a digoxigenin-labeled riboprobe using the method as described (Long and Barton, 2000; http://www.wisc.edu/genetics/CATG/barton/protocols.html). PCR was conducted on *GaMYB2* cDNA to generate a template for the probe, which was complementary to the 3′ region of *GaMYB2* transcript downstream of the conserved MYB domain.

The seed trichome of transgenic Arabidopsis plants expressing *35S*::*GaMYB2* was examined under a JSM-6360LV scanning electron microscope (JEOL, Tokyo, Japan).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY641990, AY626160, AY633559, and AY626159 for the *GaRDL1*, *GaMYB2* (*FIF1*), *GaMYB109*, and *GhHOX3* cDNA clones, respectively. AY633558 is the accession number for the *RDL1-P3* promoter.

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