SCIENCE CHINA
Life Sciences

THEMATIC ISSUE: Cotton genome plus
• RESEARCH PAPER •

February 2016 Vol.59 No.2: 194–205
doi: 10.1007/s11427-015-4991-4

# Cotton *GhMYB7* is predominantly expressed in developing fibers and regulates secondary cell wall biosynthesis in transgenic *Arabidopsis*

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Received May 14, 2015; accepted July 20, 2015; published online January 22, 2016

The secondary cell wall in mature cotton fibers contains over 90% cellulose with low quantities of xylan and lignin. However, little is known regarding the regulation of secondary cell wall biosynthesis in cotton fibers. In this study, we characterized an R2R3-MYB transcription factor, *GhMYB7*, in cotton. *GhMYB7* is expressed at a high level in developing fibers and encodes a MYB protein that is targeted to the cell nucleus and has transcriptional activation activity. Ectopic expression of *GhMYB7* in *Arabidopsis* resulted in small, curled, dark green leaves and also led to shorter inflorescence stems. A cross-sectional assay of basal stems revealed that cell wall thickness of vessels and interfascicular fibers was higher in transgenic lines overexpressing *GhMYB7* than in the wild type. Constitutive expression of *GhMYB7* in *Arabidopsis* activated the expression of a suite of secondary cell wall biosynthesis-related genes (including some secondary cell wall-associated transcription factors), leading to the ectopic deposition of cellulose and lignin. The ectopic deposition of secondary cell walls may have been initiated before the cessation of cell expansion. Moreover, GhMYB7 was capable of binding to the promoter regions of *AtSND1* and *AtCesA4*, suggesting that GhMYB7 may function upstream of NAC transcription factors. Collectively, these findings suggest that GhMYB7 is a potential transcriptional activator, which may participate in regulating secondary cell wall biosynthesis of cotton fibers.

**cotton (*Gossypium hirsutum*), fiber development, MYB transcription factor, secondary cell wall (SCW) biosynthesis, ectopic gene expression**

**Citation:** Huang, J., Chen, F., Wu, S., Li, J., and Xu, W. (2016). Cotton *GhMYB7* is predominantly expressed in developing fibers and regulates secondary cell wall biosynthesis in transgenic *Arabidopsis*. Sci China Life Sci 59, 194−205. doi: 10.1007/s11427-015-4991-4

## INTRODUCTION

Cotton fibers, the world's most important natural textile material, are single-cell protrusions originating from individual epidermal cells of the ovules. Fiber development can be divided into four distinct but overlapping phases, that is, fiber initiation, primary cell-wall formation, secondary cell-wall thickening, and maturation (Basra and Mailk, 1984). The secondary cell wall (SCW) of the mature fiber consists of over 90% cellulose, with low quantities of xylan and lignin (Haigler et al., 2012; Han et al., 2013). Deposition of >90% cellulose in cotton fiber cell walls during the SCW thickening stage makes this unique cell valuable in understanding the regulation of plant cell wall biogenesis. However, little is known about how SCW formation of cotton fibers is regulated by transcription factors (TFs), including MYB TFs. Currently, only one study has elucidated the role of a MYB protein in SCW biosynthesis of cotton fibers (Sun et al., 2015).

Plant R2R3-MYB proteins constitute one of the largest

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TF families. A large number of R2R3-MYB protein genes have been identified in monocotyledonous and dicotyledonous plant genomes. For example, there are 126 members of the R2R3-MYB family in *Arabidopsis*, 202 members in poplar (*Populus trichocarpa)*, 125 members in rice (*Oryza sativa*), and 204 members in *Gossypium raimondii* (Paterson et al., 2012; Zhao and Dixon, 2011). Global gene expression analysis revealed that numerous R2R3-MYB TFs are expressed in cotton fibers. Previously, however, most studies have focused on the roles of MYBs, such as *GhMYB2*, *GhMYB25*, *GhMYB25-like*, and *GhMYB109*, in fiber initiation and elongation (Guan et al., 2014; Machado et al., 2009; Pei, 2015; Pu et al., 2008; Walford et al., 2011; Wang et al., 2012). Recently, the function of R2R3-MYBs in controlling SCW biosynthesis has attracted significant attention due to the great importance of plant cell walls with respect to the production of sustainable biofuels. *Arabidopsis* is an extensively studied model dicot, and many studies of this plant genus have shown that numerous R2R3-MYBs are part of the complex regulatory network regulating SCW formation. For instance, *AtMYB26* is expressed only in *Arabidopsis* inflorescences and its transfer DNA (T-DNA) mutant produces sterile anthers. On the other hand, overexpression of *AtMYB26* causes ectopic secondary thickening and lignification, particularly in the epidermal cells of leaves, petals, and ovules. However, no ectopic deposition has been observed in roots. It seems that AtMYB26 specifically regulates SCW deposition in the endothecium (Yang et al., 2007). It has been demonstrated that *Arabidopsis MYB103* is a direct target of NST1, NST2, SND1, MYB46, and MYB83, and can also bind to the *CesA8* promoter directly. Recently, MYB103 was shown to be required for S-lignin biosynthesis in *Arabidopsis* inflorescence stems (Ohman et al., 2013; Zhong et al., 2008). Similarly, AtMYB58 is a specific transcriptional activator of lignin biosynthesis. Expression of *AtMYB58* is regulated by AtMYB46 (Zhou et al., 2009). To date, 17 *Arabidopsis* R2R3-MYBs have been identified and are known to play important roles in the regulation of SCW formation (Zhao et al., 2014; Zhong et al., 2014).

GhMYB7 was originally found to be involved in the transcriptional regulation of the lipid transfer protein gene, *LTP3*, in cotton fibers (Hsu et al., 2005). In this study, we provide direct evidence that GhMYB7 is implicated in the regulation of SCW biosynthesis. We show that *GhMYB7* is preferentially expressed in developing fibers. Constitutive expression of *GhMYB7* in *Arabidopsis* activated the expression of a suite of SCW biosynthesis-related genes and, consequently, induced ectopic deposition of SCW in transgenic plants. In addition, the yeast one-hybrid assay demonstrated that GhMYB7 can bind to the promoter regions of *AtSND1* and *AtCesA4*. Our results suggest that GhMYB7 may act as a transcriptional activator by participating in the regulation of SCW formation in cotton fibers.

## RESULTS

### Characterization of GhMYB7

Sequence alignment showed that the GhMYB7 amino acid sequence shared high similarity with AtMYB26 and AtMYB103, which have been shown to positively regulate SCW biosynthesis in *Arabidopsis* (Yang et al., 2007; Zhong et al., 2008). Furthermore, a neighbor-joining phylogenetic tree containing 10 other known cotton MYBs and 17 secondary cell wall-related *Arabidopsis* MYBs indicated that GhMYB7 is most closely related to AtMYB26 and AtMYB103 (Figure S1A in Supporting Information). Also, we employed interactive surveyed conserved motif alignment diagram and the associating dendrogram (SALAD) analysis which emphasizes conserved protein function. As shown in Figure S1B, GhMYB7 is grouped with AtMYB26, AtMYB103, AtMYB4, AtMYB61, and two functionally redundant key regulators of SCW formation (AtMYB46 and AtMYB83) in the SALAD dendrogram, suggesting that GhMYB7 may have functions similar to those of AtMYB26 and AtMYB103, and could potentially be involved in the regulation of SCW biosynthesis. Quantitative RT-PCR analysis revealed that *GhMYB7* is expressed at high levels in developing fibers (Figure 1A), suggesting that it may function in fiber development.

### The GhMYB7 protein is targeted to the cell nucleus and is a transcriptional activator

To investigate if GhMYB7 functions as a TF, the enhanced green fluorescent protein (eGFP)-tagged *GhMYB7* gene was expressed in *Arabidopsis*. eGFP fluorescence in 5-day-old root cells from the transgenic seedlings was observed under a Leica confocal laser scanning microscope. As shown in Figure 1B, GhMYB7 was targeted to the cell nucleus, reinforcing its probable role as a putative TF. Further transcriptional activation analysis indicated that GhMYB7 could also activate expression of the *His3*, *Ade* and *β-Gal* reporter genes in yeast (Figure 1C), suggesting that GhMYB7 may be a transcriptional activator.

### Overexpression of *GhMYB7* in *Arabidopsis* causes ectopic deposition of SCWs in various tissues

To investigate whether GhMYB7 participates in the regulation of SCW biosynthesis, we expressed *GhMYB7* in *Arabidopsis* driven by the CaMV 35S promoter. Among the 32 transgenic lines obtained, 21 exhibited small curled leaves and shorter inflorescence stems. Two independent transgenic lines overexpressing *GhMYB7* (T2 generation) were selected for further study because of the high expression level of *GhMYB7* in these plants (Figure 2). Toluidine blue staining of hand-cut stem sections showed that many epidermal cells in stems from the transgenic lines were stained blue, whereas wild-type epidermal cells were stained pink (Figure 3A–C). UV illumination revealed ectopic lignin autofluorescence in epidermal cells of transgenic plant

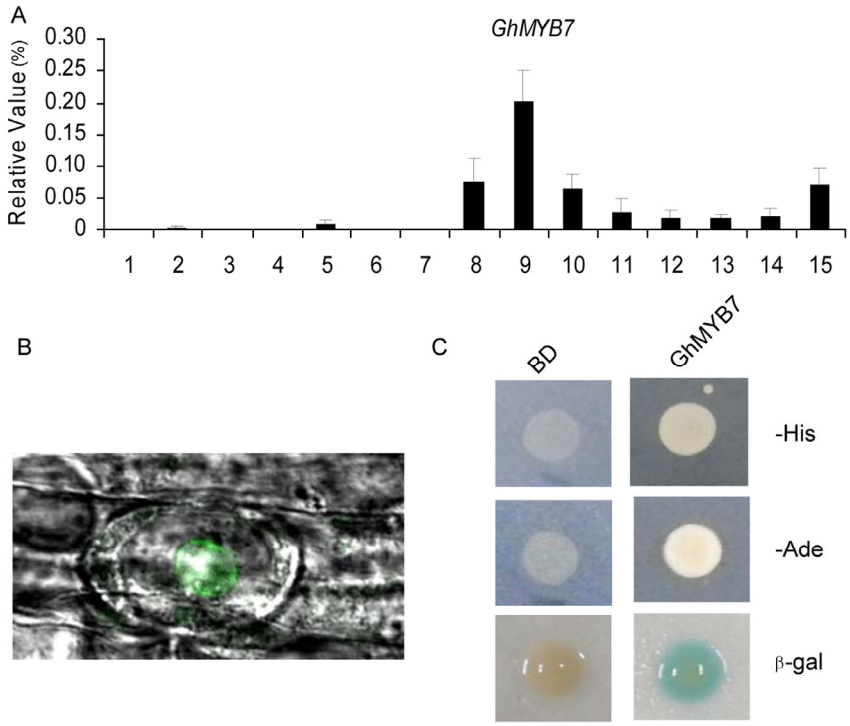


**Figure 1** Characterization of *GhMYB7*. A, Quantitative PCR analysis of *GhMYB7* expression in different cotton tissues. 1: roots; 2: hypocotyls; 3: cotyledons; 4: leaves; 5: stems; 6: petals; 7: anthers; 8: 10 dpa ovules; 9: 0 dpa fibers; 10: 3 dpa fibers; 11: 6 dpa fibers; 12: 10 dpa fibers; 13: 15 dpa fibers; 14: 18 dpa fibers; 15: 20 dpa fibers. dpa: day post-anthesis. B, Subcellular localization of GhMYB7 in *Arabidopsis* seedling roots. Green fluorescence accumulated in the cell nucleus. C, Transcriptional activation analysis of GhMYB7 in yeast. GhMYB7 was capable of activating expression of *His*, *Ade,* and *β-Gal* reporter genes.

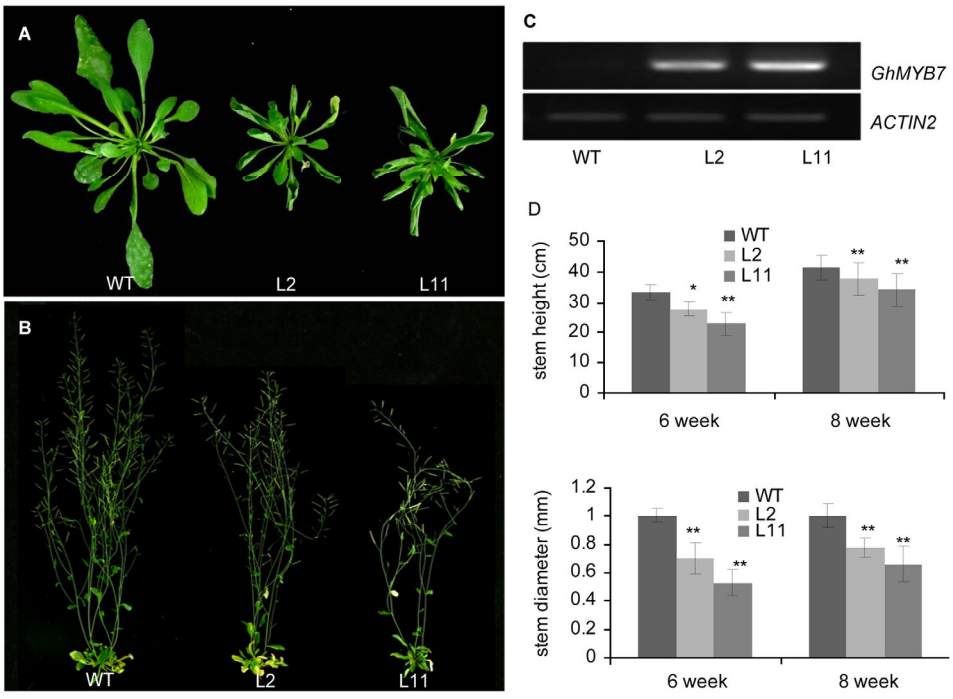


**Figure 2** Whole plant morphologies of wild-type *Arabidopsis* and two independent transgenic lines overexpressing *GhMYB7*. A, Four-week-old seedlings of wild-type *Arabidopsis* and the *GhMYB7* transgenic lines. B, Eight-week-old plants of wild-type *Arabidopsis* and the *GhMYB7* transgenic lines. C, RT-PCR analysis of *GhMYB7* expression in wild-type and *GhMYB7* transgenic lines. D, Descriptive statistics for inflorescence stem height and diameter of 6-week-old and 8-week-old wild-type and transgenic plants. Mean±SD inflorescence stem height (cm) and diameter (mm) ($n$=20 plants). Asterisks represent significant differences ($P$<0.01) between the wild-type and *GhMYB7* transgenic lines. WT: wild type; L2 and L11: *GhMYB7* transgenic lines.

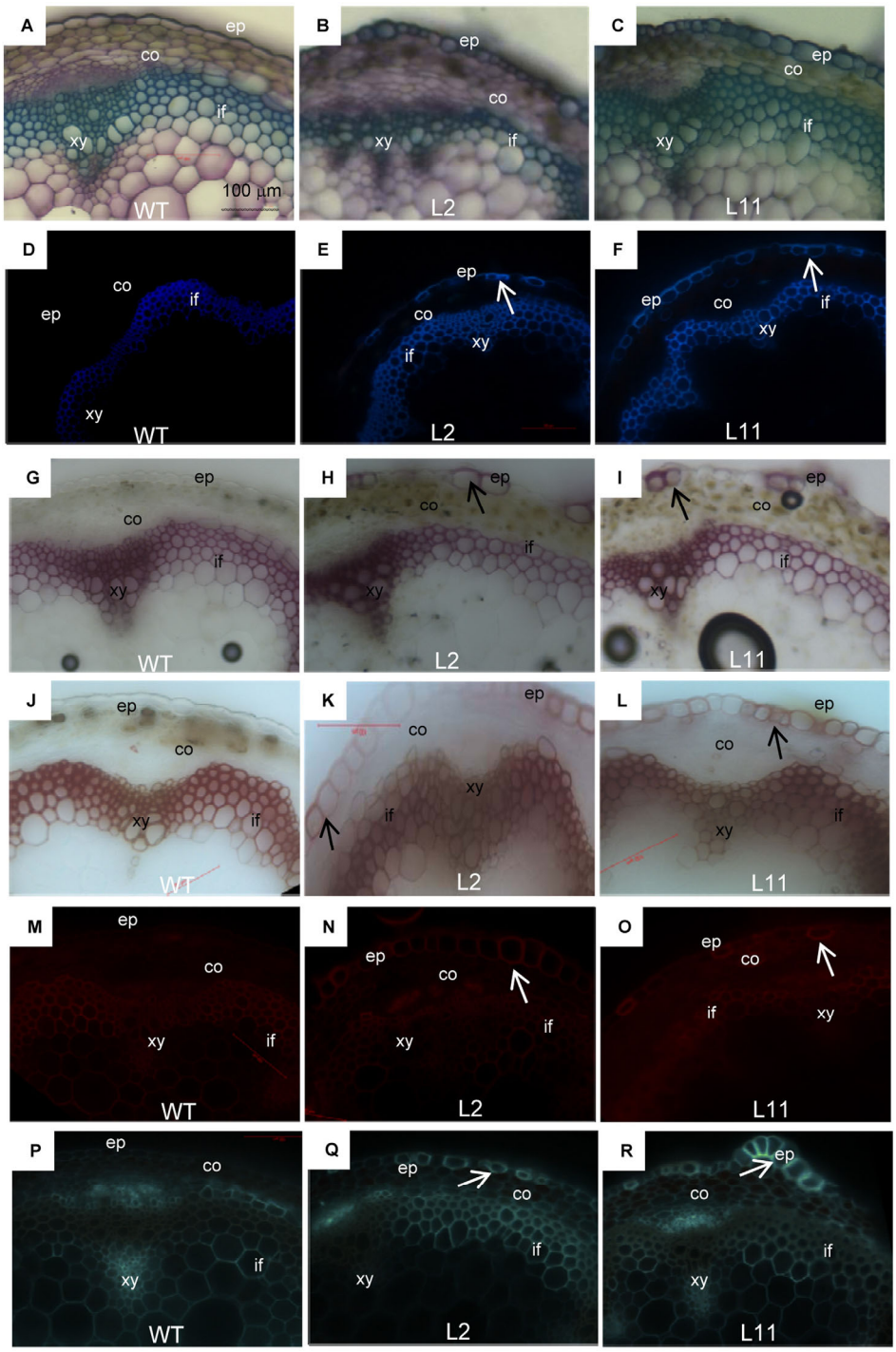


**Figure 3** Overexpression of *GhMYB7* results in ectopic deposition of secondary cell wall components in epidermal cells of transgenic *Arabidopsis* stems. A–C, Hand-cut cross-sections of stems stained with toluidine blue. D–F, UV-autofluorescence of stem cross-sections. G–I, Phloroglucinol staining of stem cross-sections. J–L, Maule staining of stem cross-sections. Arrows denote areas of ectopic lignin deposition. M–O, Stem cross-sections stained with S4B. P–R, Stem cross-sections stained with Calcofluor white. Arrows denote areas of ectopic cellulose deposition. xy: xylem; if: interfascicular fibers; co: cortex; ep: epidermis; WT: wild type; L2 and L11: *GhMYB7* transgenic lines.

stems overexpressing *GhMYB7*, but not in wild-type epidermal cells (Figure 3D–F). Similarly, phloroglucinol and Maule staining also verified ectopic lignin deposition in epidermal cells of the *GhMYB7* transgenic stems, but no lignin was detected in stem epidermal cells of wild-type plants (Figure 3G–L).

Next, we examined if overexpression of *GhMYB7* caused ectopic deposition of SCW cellulose in transgenic *Arabidopsis*. Calcofluor white, Congo red, and S4B were used to image the cellulose distribution in plant cell walls (An-

derson et al., 2010; Pradhan et al., 2014). As shown in Figure 3M–R, histological staining of cellulose in stems of the transgenic lines revealed that ectopic deposition of cellulose occurred in *GhMYB7* transgenic stem epidermal cells, whereas SCW thickening was not detected in stem epidermal cells of wild-type plants.

Further detailed analysis of paraffin-embedded stem sections revealed that overexpression of *GhMYB7* in *Arabidopsis* resulted in a significant increase in cell wall thickness of interfascicular fibers and vessels. The cell wall thickness of vessels and interfascicular fibers in the two *GhMYB7* transgenic lines was up to 15% and 30% higher than that in wild-type plants (Figure 4). Similar to the hand-cut sections showing ectopic lignification in epidermal cells of the transgenic stems, staining of paraffin-embedded stem sections with S4B revealed stronger fluorescence signals in epidermal cells of transgenic stems, whereas no signal was detected in wild-type epidermal cells (Figure S2G–I in Supporting Information). The cellulose-directed CBM3a was also employed to detect cellulose in the lignified epidermis. As shown in Figure S2J–L, noticeable labeling with CBM3a was observed for some epidermal cells of the transgenic lines, but faint labeling was observed for the wild-type epidermis. Furthermore, overexpression of *GhMYB7* caused remarkable elevation (up to 15%) of the crystalline cellulose content in stems of *GhMYB7* transgenic plants (Figure 5).

By contrast, the patchy ectopic deposition of SCW lignin and cellulose occurred in the cortex, but not the epidermis of *GhMYB7* transgenic roots (Figure 6), a result that differed from the findings observed in stems. It is notable, however, that overexpression of *GhMYB7* results in SCW deposition in the epidermis of stems, but barely induces SCW thickening in the cortex of stems. By contrast, *GhMYB7* overexpression resulted in ectopic deposition of SCWs in cortical cells of roots, but not in the root epidermis. This result may be ascribable to the differential responses of roots and stems to *GhMYB7* expression. Taken together, overexpression of *GhMYB7* in *Arabidopsis* resulted in ectopic SCW thickening in various tissues of the transgenic plants.

### Overexpression of *GhMYB7* in *Arabidopsis* activates a number of SCW-related genes

To investigate whether expression of the SCW-related genes is altered in *GhMYB7* transgenic plants, RT-PCR analysis was performed using RNA from 4-week-old seedlings of wild-type and transgenic lines overexpressing *GhMYB7*. Our results demonstrated that the transgenic lines had substantially higher transcript levels of the genes involved in SCW cellulose (*CesA4*, *CesA7*, and *CesA8*) and lignin biosynthesis (*4CL1*, *CCoAOMT1*, and *PAL1*) (Figure S3 in Supporting Information) than wild-type plants. This finding indicates that ectopic SCW thickening started before

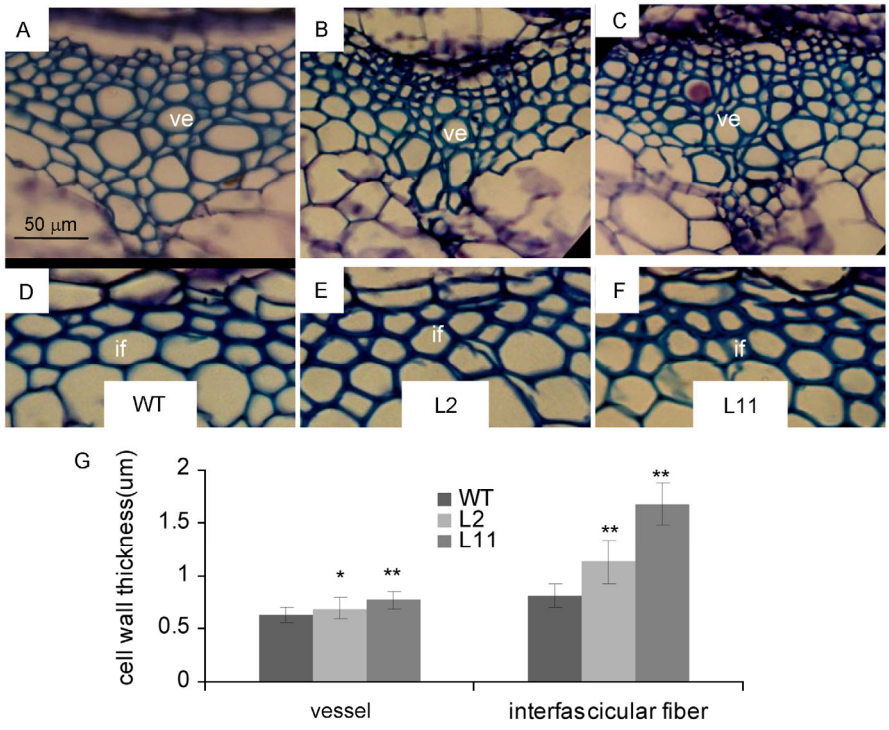


**Figure 4** Transverse sections of basal stems embedded in paraffin and stained with toluidine blue from wild-type *Arabidopsis* and two transgenic lines overexpressing *GhMYB7*. A–C, Vascular bundles. D–F, Interfascicular fibers. xy: xylem; if: interfascicular fibers. G, Average wall thickness of xylem vessels and interfascicular fibers in stems of wild-type *Arabidopsis* and two transgenic lines overexpressing *GhMYB7*. Micrographs were analyzed using ImageJ by measuring cell wall thickness. Data are mean (μm)±SD from 20 cells and three different stems per genotype. The asterisk represents a significant difference between the wild-type and transgenic lines. WT: wild type; L2 and L11: *GhMYB7* transgenic lines.

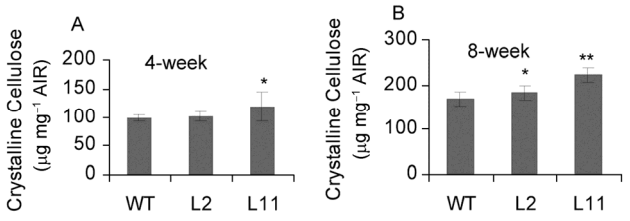


**Figure 5** Assay of crystalline cellulose content from the cell wall in *GhMYB7* transgenic *Arabidopsis*. Crystalline cellulose content in the cell wall was determined in 4-week-old *Arabidopsis* seedlings (A) and 8-week-old stems (B). Crystalline cellulose content was enhanced in the *GhMYB7* transgenic lines relative to the wild type. Error bars represent the SD of three replicates. One or two asterisks indicate significant differences at alpha values of 0.05 and 0.01, respectively. WT: wild type; L2 and L11: *GhMYB7* transgenic lines.

cessation of cell elongation, which hindered plant growth and led to dwarfs. In the stems of 8-week-old plants, we observed that overexpression of *GhMYB7* activated genes involved in SCW cellulose and lignin biosynthesis. Although we did not analyze xylan, we observed that expression of xylan synthesis genes was also significantly upregulated in transgenic plants (Figure 7). These results suggest that GhMYB7 is a transcriptional activator capable of regulating the entire process of SCW biosynthesis. Moreover, expression of SCW-related *TF* genes (including *AtNST1*, *AtNST2*, *AtSND1*, *AtMYB20*, *AtMYB46*, and *AtMYB58*) was remarkably induced, whereas *AtMYB52* was markedly downregulated in *GhMYB7* transgenic plants. Our results indicate that overexpression of *GhMYB7* in *Arabidopsis* causes a significant elevation in the expression of SCW biosynthetic genes, thereby leading to the ectopic deposition of SCW in the transgenic plants.

### GhMYB7 binds to the promoters of the *AtSND1* and *AtCesA4* genes

AC-rich and AC-like *cis*-elements are considered to be bound by MYB proteins (Kim et al., 2013; Zhao and Dixon, 2011; Zhong et al., 2013). We used the eight functional variants of AC-like motifs to BLAST search the promoters of the genes examined above and found that two to three copies of the *cis*-element variants were present in promoters of *AtSND1* and *AtCesA4*. Then, we performed a yeast one-hybrid assay to test whether GhMYB7 binds to these promoters. As shown in Figure 8, GhMYB7 could bind to *AtCesA4* and *AtSND1* promoters. Our results potentially indicate that, after binding to AC-like *cis*-elements, GhMYB7 activates expression of downstream target genes, such as *AtSND1* and *AtCesA4*.

## DISCUSSION

Cotton is an ideal experimental system for studying cell expansion and cell wall biosynthesis (Kim and Triplett, 2001). Mature cotton fiber has a unique SCW structure that is composed almost entirely of cellulose (>90%) and is distinct from that of all other known plant species. By contrast, typical SCWs in dicotyledonous stem xylem contain 40%–50% cellulose. Therefore, understanding fiber SCW formation better enables modification of the cell wall for improvements in fiber quality and quantity. Previous studies reported that some cell wall proteins and their related enzymes (e.g., AGPs, PRPs, and glycosyltransferases) are involved in SCW formation of cotton fibers (Huang et al., 2013; Li et al., 2014; Sun et al., 2015; Xu et al., 2013). Ten members belonging to the cellulose synthase (CesA) family have been identified in cotton, of which CesA1, CesA2, CesA7, and CesA8 are implicated in SCW formation in fibers (Li et al., 2013; Li et al., 2015). Recently, a study indicated that a cotton LIN-11, Isl1 and MEC-3(LIM)-domain protein, WLIM1a, increases lignin content, leading to improvement in both fiber fineness and strength, which means that altering cell wall composition can change fiber quality, although fiber is composed of only a small quantity of lignin (Han et al., 2013). However, the authors also posited that lignin biosynthesis in fiber must be controlled at an optimal level or the fiber might become rigid, which is not favorable for processing (Han et al., 2013). Therefore, a thorough understanding of how fiber SCW biosynthesis is regulated may provide a mechanism for fine-tuning cotton cell wall composition.

Several studies have shown that the SCW transcriptional network is widely conserved in different plant species. In *Arabidopsis*, NAC-domain TFs (including NSTs and VNDs) acting at the top tier direct a transcriptional network involving MYB TFs, ultimately inducing the expression of structural genes involved in SCW biosynthesis. A similar cascade involving NACs and MYBs has been found in poplar (*Populus trichocarpa*), pine (*Pinus taeda*), maize (*Zea mays*), and rice (*Oryza sativa*) (Duval et al., 2014; Zhong et al., 2011; Zhong et al., 2013). More than 10 MYB TFs have been identified in Upland cotton. However, the vast majority are involved in fiber initiation and elongation, but not in SCW formation. For example, six MYB-domain genes, *GhMYB1* to *GhMYB6*, were expressed in cotton ovules at -3 dpa, the stage at which fiber primordia differentiate (Cedroni et al., 2003; Loguercio et al., 1999). Transcripts of *GhMYB8* and *GhMYB10* were more abundant in flowers and roots and less abundant in leaves and fibers. Overexpression of *GhMYB10* in tobacco resulted in abnormal cell shapes in leaf trichomes, suggesting that GhMYB10 might function in epidermal cell differentiation (Hsu et al., 2008). GhMYBL1 has been shown to be involved in the regulation of SCW formation only recently (Sun et al., 2015). In this study, we further characterized an *R2R3-MYB* gene, *GhMYB7*, which has previously been shown to regulate the *Ltp3* gene, participate in gibberellin (GA) biosynthesis, and regulate the GA signaling pathway during fiber development (Hsu et al., 2005). We found that GhMYB7 exhibited high similarity with two extensively characterized *Arabidopsis* SCW-associated MYB TFs, AtMYB26 and

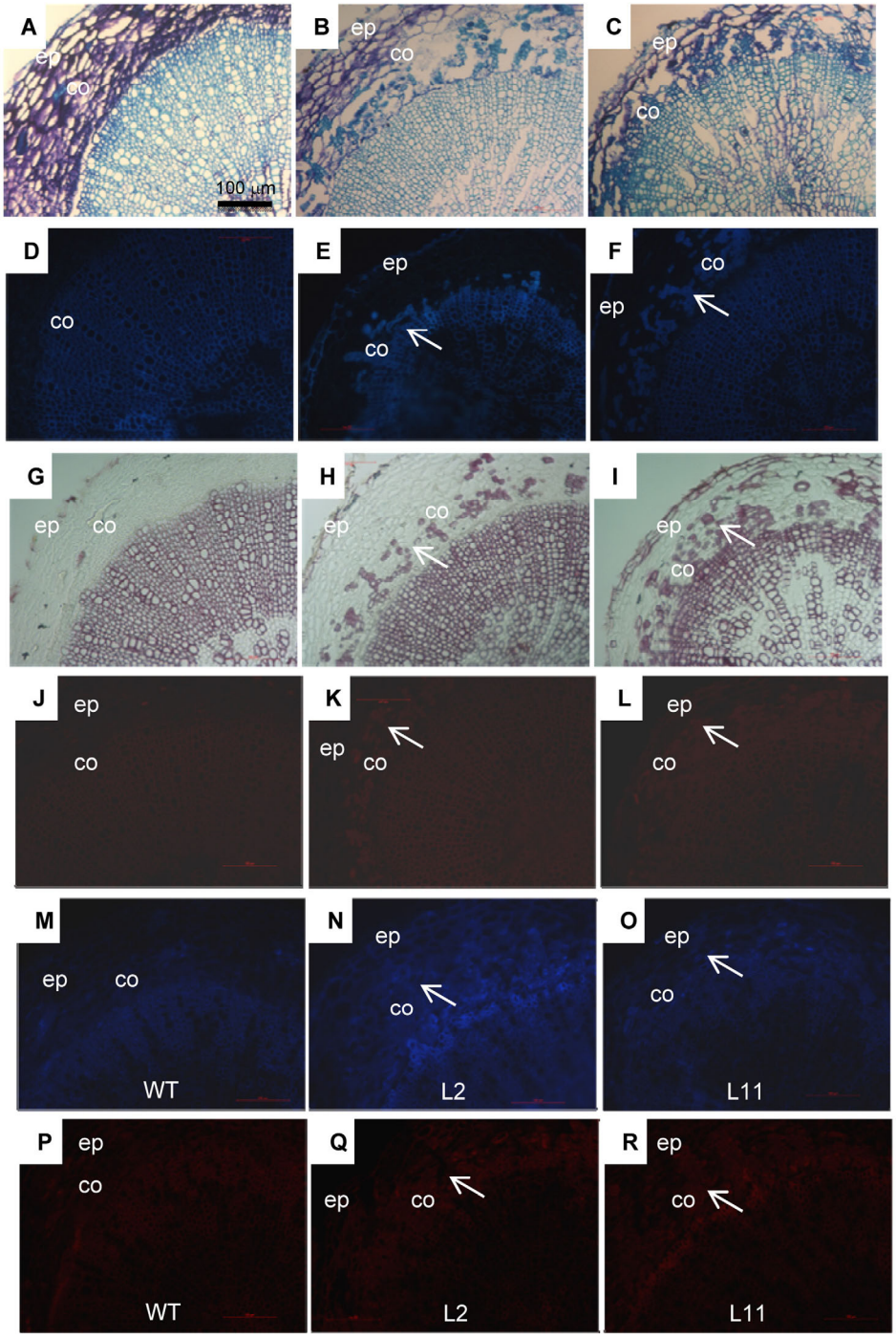


**Figure 6** Overexpression of *GhMYB7* induces ectopic deposition of secondary cell wall components in the cortex of primary roots in transgenic *Arabidopsis*. A–I, Wild-type root cross-sections (left) and *GhMYB7* transgenic line (L2 and L11) root cross-sections stained with toluidine blue (A–C), analyzed on the basis of UV-autofluorescence (D–F), or stained with phloroglucinol HCl (G–I). Arrows indicate areas of ectopic lignin deposition in the cortex. J–R, Transverse sections of roots stained with Pontamine Fast Scarlet 4B (J–L), Calcofluor white (M–O), and Congo red (P–R). Arrows indicate areas of ectopic cellulose deposition in the cortex. ep: epidermis; co: cortex; WT: wild type; L2 and L11: *GhMYB7* transgenic lines.

AtMYB103. Considering that the transcriptional regulators in SCW biosynthesis are evolutionarily conserved between monocots and dicots, this finding prompted us to hypothesize that GhMYB7 is likely to participate in the control of SCW biosynthesis. We present three lines of evidence to support this hypothesis. First, we overexpressed *GhMYB7* in *Arabidopsis* to determine its functional role in the regulation of SCW formation. This method has been successfully employed previously to investigate the functions of MYBs in rice and maize (Zhong et al., 2011). Our results revealed

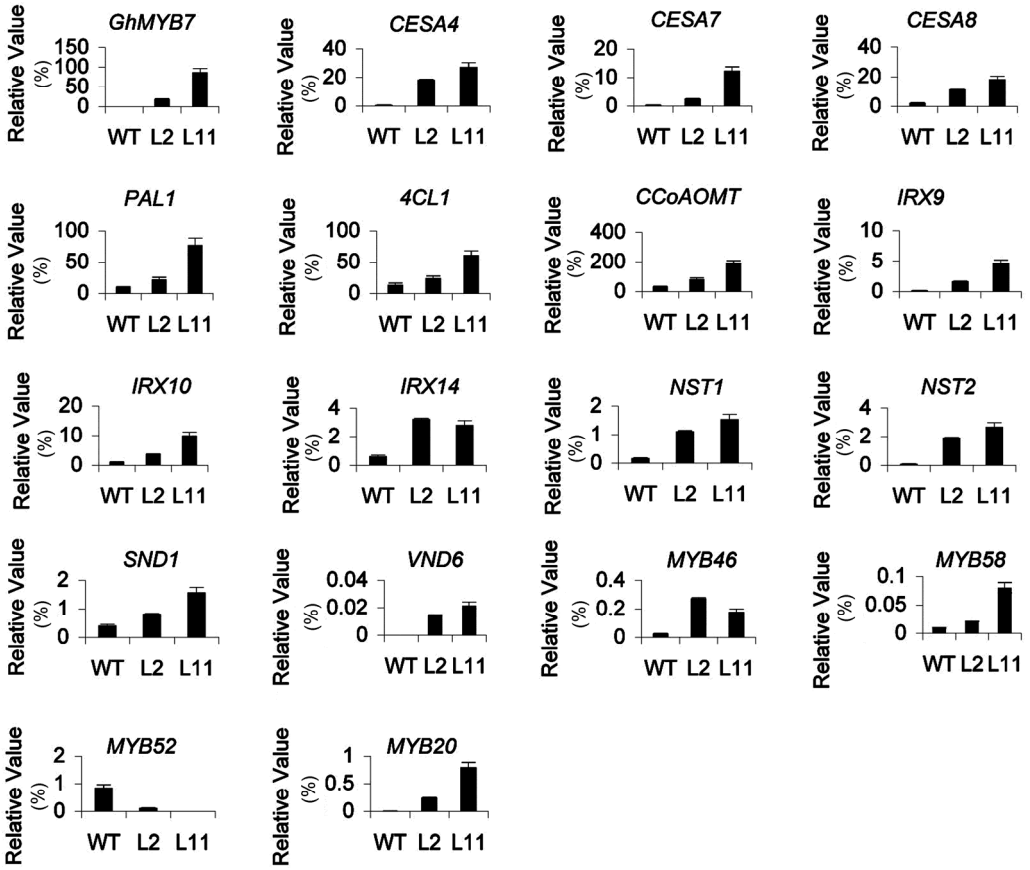


**Figure 7** Quantitative RT-PCR analysis of gene expression related to secondary cell wall biosynthesis in *GhMYB7* transgenic *Arabidopsis*. Total RNA was isolated from 8-week-old inflorescence stems of wild-type transgenic *Arabidopsis*. The *Arabidopsis ACTIN2* gene was used as a standard control. Error bars represent the SD of three replicates. WT: wild type; L2 and L11: *GhMYB7* transgenic lines.

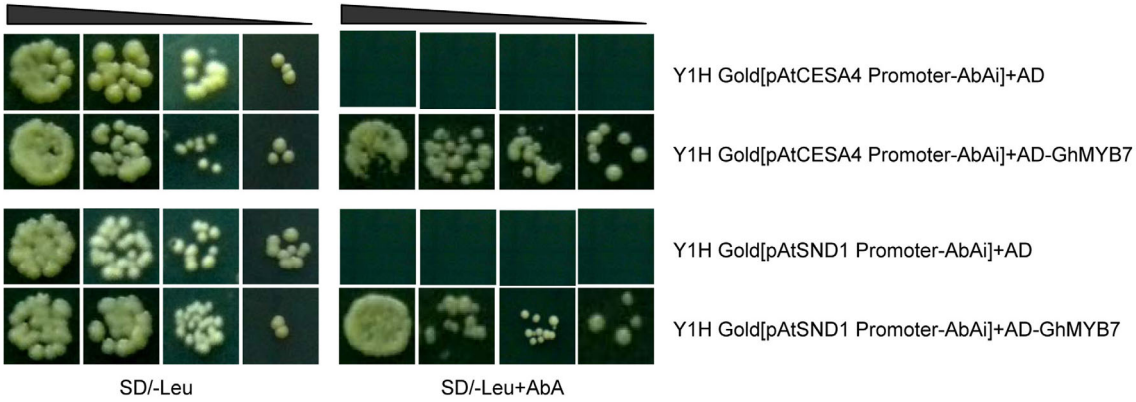


**Figure 8** Interaction between GhMYB7 protein and promoter sequences of *AtCesA4* and *AtSND1* in the yeast one-hybrid system. Yeast cells (10 μL) were subjected to serial dilutions (1, 0.1, 0.01, and 0.001) and spotted onto a synthetic dropout medium lacking leucine, with or without 400 ng $mL^{-1}$ AbA. The AD protein served as a negative control.

that overexpression of *GhMYB7* resulted in ectopic deposition of SCW in the transgenic plants. Second, we observed that the expression of genes involved in biosynthesis of SCW components was significantly upregulated in *GhMYB7* transgenic lines. A number of SCW-related TF genes were also induced, and it should be noted that *AtMYB52* expression was significantly suppressed. A recent study revealed that ectopic lignin deposition was detected in the epidermal cells of a *myb52* T-DNA insertion mutant, suggesting that AtMYB52 is likely to be a transcriptional repressor of lignin biosynthesis or the entire process of SCW formation (Cassan-Wang et al., 2013). Likewise, our

work also supports the role of AtMYB52 as a repressor of the entire process of SCW formation, although Zhong et al. proposed AtMYB52 as an activator of SCW biosynthesis (Zhong et al., 2011). Third, our yeast one-hybrid assay demonstrated that GhMYB7 could directly bind to the promoters of *AtSND1* and *AtCesA4*.

In *Arabidopsis*, NST1, SND1, and VND1-VND7 are phylogenetically clustered in one subgroup of the NAC family and are implicated as "master switches" of SCW biosynthesis. *NST1* and *SND1* are fiber-specific, while *VND1-VND7* are vessel-specific. Analyses of mutant phenotypes showed that NST1 and SND1 control SCW development in interfascicular and vascular fibers, while VND1-VND7 control SCW development in stem and root vessels. In agreement with their distinct expression patterns, these results imply the spatial specificity of some SCW-related TFs. Our research showed that *GhMYB7* overexpression caused ectopic SCW deposition not only in epidermal cells of stems but also in the cortex of roots, a result in contrast with those for the two closest *Arabidopsis* homologs, AtMYB103 and AtMYB26. These results suggest that cotton fiber-related MYB may have spatial specificity.

Previous studies in different plant species have shown that functionally redundant *MYB46* and *MYB83* are direct targets of the two NACs, SND1 and NST3 (Duval et al., 2014; Shang et al., 2013; Zhong et al., 2011; Zhong et al., 2013). However, Yang et al. found that overexpression of *AtMYB26* induced the expression of *NST1* and *NST2*, suggesting that AtMYB26 may act upstream from these two NAC genes in anther endothecium (Yang et al., 2007). More recently, Endo et al. identified 14 members of multiple TF families, including GATA12, ANAC075, and VND1-VND5, as putative upstream regulators of *VND7* expression (Endo et al., 2014). In this study, our data clearly showed that, regardless of plant age or tissue type, GhMYB7 can activate the expression of both *AtNST1* and *AtSND1*, suggesting that GhMYB7 might also act upstream of NAC TFs (Figure S2 in Supporting Information, Figure 8). However, additional research is required to confirm this presumption.

Hsu et al. found that overexpression of *GhMYB7* in both tobacco and *Arabidopsis* resulted in dwarf plants, abnormal leaf shape, and retarded root development (Hsu et al., 2005). The authors deemed that GhMYB7 might play a role in the transcriptional regulation of the *Ltp3* gene and participate in either GA biosynthesis or the GA signaling pathway during fiber development (Hsu et al., 2005). In this study, we further showed that GhMYB7 may play a role in the regulation of SCW formation. Our findings, together with Hsu's results, suggest that SCW synthesis is tightly integrated with other developmental processes and signaling pathways. In summary, overexpression of *GhMYB7* in *Arabidopsis* implies potential regulatory roles in SCW biosynthesis in cotton fibers. Although this study cannot rule out the possibility that GhMYB7 may function differently in cotton fibers, our results provide a solid basis for future research deciphering the roles of *GhMYB7* in cotton fiber development. We are now employing overexpression and dominant repression of *GhMYB7* in cotton to elucidate its roles in detail.

## MATERIALS AND METHODS

### Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (ecotype *Columbia*) were surface-sterilized with 75% ethanol for 1 min and 5% NaClO for 3 min, followed by washing with sterile distilled water. Sterilized *Arabidopsis* seeds were sown on half-strength Murashige-Skoog (MS) media (10 g $L^{-1}$ sucrose, pH 5.8) maintained at 4°C for 48 h. The plates were then transferred to a plant growth chamber (Sanyo, Osaka, Japan) for 7 d before transplantation into soil (16 h light/8 h dark, 22±1°C).

Cotton (*Gossypium hirsutum* cv. Coker 312) seeds were surface-sterilized with 75% (v/v) ethanol for 1 min and 10% (v/v) $H_2O_2$ for 2 h, followed by washing with sterile distilled water. Sterilized cotton seeds were sown on half-strength MS media (16 h light/8 h dark, 28°C) for 5 d, and then transplanted into soil for growth to maturation.

### RNA extraction and RT-PCR analysis

Four-week-old seedlings and 8-week-old inflorescence stems of *Arabidopsis* from the wild-type and transgenic lines were used for total RNA extraction as described previously (Li et al., 2014). Different cotton tissues (including roots, hypocotyls, cotyledons, leaves, stems, petals, anthers, ovules, and fibers) were used for total RNA isolation (Li et al., 2002). Quantitative RT-PCR analysis was conducted as described previously (Li et al., 2005).

### Subcellular localization and transcriptional activation analysis

The *GhMYB7* coding sequence was cloned into the binary vector pBI121. Then, the enhanced green fluorescent protein (eGFP) coding region was cloned into the same vector downstream of the *GhMYB7* coding sequence. The construct was introduced into *Arabidopsis* by the floral dip method. The localization pattern of the *GhMYB7* protein in the transgenic *Arabidopsis* root cells was visualized under a Leica TCS SP5 confocal laser scanning fluorescence microscope (Leica, Germany). For transcriptional activation analysis, the *GhMYB7* coding region was fused with the GAL4-DNA binding domain (GAL4-BD) and was subsequently expressed in yeast; it was then examined for activation of the expression of the *His3*, *Ade*, and *β-Gal* reporter genes.

### Sectioning of stems and roots for microscopic analysis

Stems were sectioned manually with a razor blade and

stained with 0.025% toluidine blue for 1 min, washed carefully in distilled water, and mounted in 10% glycerol solution.

Paraffin-embedded stems and roots were sectioned into 3- to 5-μm-thick slices under a Leica microtome using a method described previously (Li et al., 2014). For lignin staining, sections were stained with either phloroglucinol HCl or Maule reagent as described previously (Pradhan et al., 2014). Positive results for phloroglucinol staining indicate the presence of guaiacyl-lignin, whereas those for Maule reagent staining indicate the presence of syringyl-lignin. For cellulose staining, three dyes were used. Briefly, sections were stained in 0.01% Calcofluor white for 30 s, washed gently with water, mounted in 50% glycerol, and observed under UV excitation. Cell wall material stained using this process should appear pale blue. Sections were stained in 0.5% Congo red for 13 min, washed three times with water, mounted in water, and observed under blue light excitation. Sections were stained for 5 min in Pontamine Fast Scarlet 4B (S4B; 0.1% S4B (w/v) in 150 mmol $L^{-1}$ NaCl), washed carefully with water, mounted in 10% glycerol, and excitation at 561 nm (Thomas et al., 2013).

### Immunolocalization of cell wall cellulose

We used cellulose-directed carbohydrate-binding module (CBM) 3a to examine the distribution and abundance of cellulose. The immunolabeling experiment was conducted as described previously (Li et al., 2014).

### Cell wall extraction and crystalline cellulose analysis

Extraction of cell wall residues (i.e., alcohol-insoluble residues [AIRs]) was conducted as described previously (Huang et al., 2013; Qin et al., 2013). Cell wall crystalline cellulose amount was measured according to Kim et al. (Kim et al., 2013). Briefly, AIRs were treated with type-I porcine α-amylase for 48 h at 25°C, followed by vigorous shaking to remove starch, and then hydrolyzed with 2 mol $L^{-1}$ trifluoroacetic acid (TFA) for 90 min at 121°C. The remaining residue was washed with Updegraff reagent ($HOAc/H_2O/HNO_3$, 8∶2∶1, v/v) to deprive hemicellulose and amorphous glucan. The air-dried residue was hydrolyzed with 60% sulfuric acid for 30 min at room temperature, and the resulting monosaccharides were quantified by the anthrone method.

### Yeast one-hybrid assay

The yeast one-hybrid assay was performed according to the Matchmaker Gold Yeast One-Hybrid System (Clontech Laboratories, Inc.) protocol. Briefly, promoter sequences (~1000 bp) of *AtCesA4* and *AtSND1* were cloned upstream of the aureobasidin A (*AbA$^r$*) gene of the pAbAi vector and transformed into Y1HGold yeast by homologous recombination to generate a reporter strain. *GhMYB7* was cloned downstream of the yeast GAL4 transcription activation domain (GAL4-AD), and the resulting pGADT7-GhMYB7 effector vector was introduced into the reporter strain. Yeast growth was recorded with, or without, inclusion of $AbA^r$. When *GhMYB7* binds to the promoter sequence, GAL4-AD activates expression of $AbA^r$, which enables the yeast cells to grow on synthetic drop-out media (without leucine) containing AbA.

**Compliance and ethics** *The author(s) declare that they have no conflict of interest.*

**Acknowledgements** *This work was supported by the National Natural Science Foundation of China (31371234), and the project from the Ministry of Agriculture of China for transgenic research (2014ZX08009-27B).*

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## SUPPORTING INFORMATION

**Figure S1** Phylogenetic analysis of GhMYB7 with secondary cell wall-related *Arabidopsis* MYB proteins.

**Figure S2** *GhMYB7* overexpression causes ectopic deposition of secondary cell wall components in stem epidermal cells of transgenic *Arabidopsis*.

**Figure S3** RT-PCR analysis of gene expression related to secondary cell wall biosynthesis in *GhMYB7* transgenic *Arabidopsis*.

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