The GhMYB36 transcription factor confers resistance to biotic and abiotic stress by enhancing PR1 gene expression in plants

Tingli Liu†, Tianzi Chen†, Jialiang Kan, Yao Yao, Dongshu Guo, Yuwen Yang, Xitie Ling, Jinyan Wang† and Baolong Zhang*

Excellence and innovation center, Jiangsu Academy of Agricultural Sciences, Nanjing, China

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*Correspondence (Tel +86 25 84390292; fax +86 25 84390297; email zhbl2248@hotmail.com).
†These authors contributed equally to this work.

Summary
Drought and Verticillium wilt disease are two main factors that limit cotton production, which necessitates the identification of key molecular switch to simultaneously improve cotton resistance to Verticillium dahliae and tolerance to drought stress. R2R3-type MYB proteins could play such a role because of their conserved functions in plant development, growth, and metabolism regulation, however, till date a MYB gene conferring the desired resistance to both biotic and abiotic stresses has not been found in cotton. Here, we describe the identification of GhMYB36, a gene encoding a R2R3-type MYB protein in Gossypium hirsutum, which confers drought tolerance and Verticillium wilt resistance in both Arabidopsis and cotton. GhMYB36 was highly induced by PEG-simulated drought stress in G. hirsutum. GhMYB36-silenced cotton plants were more sensitive to both drought stress and Verticillium wilt. GhMYB36 overexpression in transgenic Arabidopsis and cotton plants gave rise to improved drought tolerance and Verticillium wilt resistance. Transient expression of fused GhMYB36-GFP in tobacco cells was able to localize GhMYB36 in the cell nucleus. In addition, RNA-seq analysis together with qRT-PCR validation in transgenic Arabidopsis and cotton plants gave rise to improved drought tolerance and Verticillium wilt resistance. Luciferase interaction assays indicated that GhMYB36 are probably bound to the promoter of PR1 to activate its expression and the interaction, which was further verified by Yeast one hybrid assay. Taken together, our results suggest that GhMYB36 functions as a transcription factor that is involved in drought tolerance and Verticillium wilt resistance in Arabidopsis and cotton by enhancing PR1 expression.

Keywords: transcription factors, drought tolerance, transgenic plants, transient expression, R2R3-type MYB proteins.

Introduction
Cotton (Gossypium hirsutum L.) is an important commercial crop providing fiber, edible oil, and biofuel worldwide. Drought stress and Verticillium wilt disease are two main factors that severely impact on cotton growth and limit fiber yield and lint quality in China. To improve drought tolerance and Verticillium resistance of cotton, overexpression of the genes encoding the desired traits has become an efficient way to accelerate breeding process (Wang et al., 2020b; Yue et al., 2012). The implementation of such a strategy has been accelerated by the recent availability of G. hirsutum genome sequences resources (Hu et al., 2019; Li et al., 2015; Zhang et al., 2015) that facilitate functional genomic studies and provide abundant valuable genes for improving the agronomic traits of cotton (Wang et al., 2020b).

The functionally identified genes that are involved in drought tolerance in cotton can be divided into two categories. One consists of functional genes that can enhance drought tolerance directly by synthesizing osmotic adjustment substances and soluble sugars; these genes include PSSS (Yoshida et al., 1995), LEA (Yu et al., 2016), and TPS (Kosmas et al., 2006). The other category includes a number of genes encoding transcriptional factors that regulate gene expression and signal transduction under drought stress, such as MYB (Butt et al., 2017; Chen et al., 2015), NAC (Gunapati et al., 2016; Shah et al., 2013), WRKY (Chu et al., 2015; Guo et al., 2011; Yan et al., 2014), AREB/ABF (Kerr et al., 2017) and bZIP (Liang et al., 2016). Research on these genes has indicated that drought tolerance in cotton is a complex trait that is controlled by multiple genes and varying depending on species, the environmental conditions, developmental stages, and nutritional status.

Verticillium wilt caused by V. dahliae Kleb (Vd) is one of the most destructive and notorious cotton vascular diseases that impose serious challenge to cotton production worldwide. Due to the vitality of resting spores and overwintering structures produced by microsclerotia, V. dahliae is difficult to be eradicated from infected fields (Wang et al., 2020b) and has not been effectively controlled in G. hirsutum that is the most widely-cultivated cotton species. Over the past few years, a number of genes involved in defense against V. dahliae have been reported, such as LKY1 and LKY2 (Gu et al., 2017), GbCYP86A1 (Wang et al., 2020a), GhWAK7A (Wang et al., 2020b), and GhWAKL (Feng et al., 2021), but reports on their actual effects are sporadic and the underlying the molecular mechanism of these genes against V. dahliae infection in cotton largely remains unraveled.

There are four classes of MYB transcription factors (TFs) based on the number of adjacent MYB repeats (Dubos et al., 2010), most common of which is R2R3-type MYB, which is the most common superfamily (Du et al., 2012; Yanhui et al., 2006). R2R3-type MYB is comprised of a superfamily of gene members that have undergone a rapid expansion through repeated gene duplication and functional divergence, notably playing conserved roles in plant development, growth, and metabolism regulation (Dubos et al., 2010; Kranz et al., 1998; Yanhui et al., 2006). Reportedly, they are also involved in plant responses to drought...
stress, as exemplified by NbPHAN, silencing of which in Nicotiana benthamiana caused sensitivity to drought stress (Huang et al., 2013), and the overexpressing a soybean gene GmMYB36 confers drought stress in Arabidopsis (Su et al., 2014). Similarly, OsMYB2 was able to confer the increased tolerance to salt, cold, and dehydration stresses in rice (Yang et al., 2012). However, this is contradicted by the report in Arabidopsis where AtMYB20 was negatively involved in adaptive response to drought stress (Gao et al., 2014). In cotton, research on MYB protein have so far been mainly focusing on fiber development, little if any is known about its role in drought response (Guan et al., 2014; Pu et al., 2008; Wan et al., 2016; Wang et al., 2004). A diploid Cotton, Gossypium raimondii, contains over 200 R2R3-type MYB genes (Paterson et al., 2012). However, only GbMYB5 and GaMYB62L were reported to be positively involved in adaptive response to drought stress (Butt et al., 2017; Chen et al., 2015), whereas whether the remaining genes are relevant to drought tolerance or other stress tolerance is far from being elucidated (Iqbal et al., 2013).

MYB36 is a R2R3-type MYB that has been known to orchestrate the Casparian strip formation in Arabidopsis, allowing the potential for improvements in water use efficiency and enhanced resistance to abiotic stresses (Kamiya et al., 2015). In this study, we identified a homolog of Arabidopsis MYB36, GhMYB36 from G. hirsutum, the expression of which was induced by polyethylene glycol (PEG) treatment, abscisic acid (ABA), and V. dahliae infection. We have further demonstrated that GhMYB36 confers drought stress tolerance and Verticillium resistance in both cotton and Arabidopsis plants by enhancing PR1 expression.

Materials and methods

Plant materials and growing conditions

Seedings of upland cotton G. hirsutum L. cv. Ao 3503 and tobacco N. benthamiana were grown in pots (340 mL) filled with soil mix (1:1, vermiculite: humus) and placed in a growth chamber with a photosynthetic photon flux density of approximately 200 mmolm\(^{-1}\)s\(^{-1}\) and 80% relative humidity (Liu et al., 2014b).

RNA extraction and quantitative real time PCR

RNA extraction and quantitative real time PCR (qRT-PCR) were performed as previously described (Liu et al., 2014b) with few modifications. Briefly, total RNA was extracted from tobacco leaves utilizing the RNA simple Total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China). First-strand cDNA was synthesized by using Invitrogen SuperScript III Reverse Transcriptase Kit (Life Technologies, Carlsbad, CA). qRT-PCR was performed using SYBR Premix Ex TaqTM II Kit (TakaRa Biotechnology, Dalian, China) in a RT-PCR thermal cycler qTOWER 2.0.2.2 (Analytik JenaAG, Jena, Germany). Each 25 μL of qRT-PCR mixture contained 10 μL of Ace2 QPCR SYBR Green Master Mix (Vazyme Biotech. Co., Ltd., Nanjing, China), 0.2 μM of each primer, and 10 ng of 1st strand cDNA synthesized above. The amplification protocol consisted of an initial denaturation step of 1 min at 95 °C, followed by 40 cycles of amplification consisting of denaturation for 15 s at 95 °C, annealing for 20 s at 60 °C, and extension for 20 s at 72 °C. The relative expression levels were calculated using the 2\(^{-\Delta\DeltaCT}\) method (Livak and Schmittgen, 2001). Data representing three biological replicates and three technical replicates are expressed as mean ± standard error (SE). The qRT-PCR primer sequences are presented in Table S1.

Virus-induced gene silencing in cotton

Virus-induced gene silencing (VIGS) was performed as previously described (Gu et al., 2014; Zhang et al., 2012). Briefly, a 214-bp DNA fragment of GhMYB36 was synthesized by PCR amplification using the primers 36VIGSF and 36VIGSR, which was inserted into pCLCVA (Gu et al., 2014), resulting in pCLCVA–GhMYB36 that was introduced into Agrobacterium tumefaciens strain LBA4404. Agro-infiltration for VIGS in cotton was performed by plants following the procedures as previously described (Gu et al., 2014; Zhang et al., 2012). The expression of GhMYB36 in the Agro-infiltrated G. hirsutum plants was determined by qRT-PCR using MYB36F and MYB36R as primers, and cotton ubiquitin 14 (UBQ14) as a reference gene (Liu et al., 2014a).

Construction of overexpression vector and plant transformation

The entire coding region of GhMYB36 was amplified by using MYB36OF and MYB36OR as primers, which was inserted into the overexpression vector pBinGFP4 (Liu et al., 2014b), behind CaMV 35S promoter. The resultant plasmid, pBinGFP4–GhMYB36, was introduced into the A. tumefaciens strain LBA4404 by electroporation, and used to transform Arabidopsis (Arabidopsis thaliana) ecotype Col-0 plants by using floral dipping method (Clough and Bent, 1998). The mature T2 seeds were placed on MS medium containing 40 mg/L of kanamycin, and the transgenic Arabidopsis plants thus selected were examined by genomic DNA PCR and RT-PCR analyses for their transgenic status, prior to plant establishment in soil. Three independent transgenic lines, OE1, OE2, and OE7, were carried through to homozygosity in T3 generation for further assessments. The expression of GhMYB36 in these plants was investigated by semi-quantitative RT-PCR (Liu et al., 2014b) using Rubi5 Co as a reference gene (de Jonge et al., 2012). The transformation of upland cotton G. hirsutum var. O3298 was conducted as previously described (Yang et al., 2015). Verticillium dahliae inoculation and quantification were performed as described previously for Arabidopsis (Fradin et al., 2011) and for cotton (Liu et al., 2014b).

Drought treatment of plants

Polyethylene glycol treatment was conducted to imitate the drought circumstance and to elucidate the function of GhMYB36 in the drought-tolerance response of cotton. Twenty days after the agro-infiltration, GbMYB36-silenced (VIGS), empty vector CLCVA-treated (EM), and WT G. hirsutum plantlets were uprooted and submerged into 18% PEG 6000 solution, and incubated for 2 days. The experiment was performed three times. Transgenic Arabidopsis seeds were surfaced-sterilized and sown on MS agar medium plates supplemented with a gradient of mannitol 0, 350, 400, 450, and 500 mm. Following a period of 3 days in the dark at 4°C for vernalization, the seeds were germinated in a growth chamber at 20°C under 16 h photoperiod and 60% relative humidity. To conduct the experiment with drought stress, 2-week-old transgenic seedlings, together with wild type (WT) Col-0, were subjected to drought treatment by withholding water until a lethal effect of dehydration was observed (more than 70% of the WT Arabidopsis were dead). The survival plants were counted and recorded by photograph.
Measurement of ion leakage and malondialdehyde content and total antioxidant activity

To assess the stability of plant cellular membranes, ion leakage was measured following the procedure described by Yu et al. (2012) using a Eutech™ CON 700 Conductivity Meter (Consort, Turnhout, Belgium). All the assays were repeated for three times, and the data were analyzed using Student’s t-test. The levels of lipids peroxidation for WT and overexpression Arabidopsis plants of WT and VIGS cotton plants were measured using a thiobarbituric acid (TBA) reaction to determine the accumulation of malondialdehyde (MDA), a major product of lipids peroxidation (Liu et al., 2014a). The supernatant was centrifuged at 12 000 g for 5 min at 4°C. A volume of 0.6 mL of the supernatant was mixed with an equal volume of 0.6% TBA. The mixture was boiled for 15 min, cooled to room temperature, and centrifuged at 12 000 g for 5 min. The supernatant was analyzed using a spectrophotometer as previously described (Liu et al., 2014a). The total antioxidant activity (T-AOC) in the leaves was assayed by using the T-AOC detection kit (Nanjing Jiancheng Bioengineering Institute Nanjing, China) following manufacturer’s instruction.

Water loss and relative water content measurements

Water loss was estimated following a method described previously by Verslues et al. (2006). Briefly, the first three fully expanded true leaves of a plant were detached and weighed, and the changes in FW over time were recorded. The rate of water loss was calculated by the loss of FW. To perform the relative water content (RWC) assay, the first fully expanded leaf was detached and its FW was immediately measured. The samples were hydrated for 12 h in distilled water to determine their turgid weight (TW), prior to drying at 65°C until constant weight was reached, which was used to estimate the dry weight (DW). The RWC was calculated according to the formula: RWC (%) = [(FW–DW)/(TW–DW)] × 100. Comparison between lines was conducted, and the significance of the differences was assessed by ANOVA-single factor analysis.

Subcellular localization analysis of transiently expressed fusion proteins

GhMYB36 was amplified using the primers MYB36OF and MYB36OR described above and inserted into pBinGFp4 (Liu et al., 2014b). The resultant overexpression construct pBinGFp4-GhMYB36 was then introduced into A. tumefaciens strain GV3101 by electroporation (Hellens et al., 2000). The subcellular localization of GhMYB36 was determined in N. benthamiana epidermal cells by A. tumefaciens infiltration (Liu et al., 2014b). GFP fluorescence was detected in the tobacco epidermal cell using a ZEISS LSM 710 confocal microscope with 20× objective lens (Zeiss Microscopy, Jena, Germany) at excitation and emission wavelengths of 488 nm and 495–530 nm, respectively (Yu et al., 2012).

Transient expression assay in Nicotiana benthamiana

For generation of GhPR1pro-Luc reporter, promoter of GhPR1 was amplified from the total DNA of G. hirsutum cv Ao 3503 using primer pairs of PR1proF and PR1-LucR; the luciferase gene Luc was amplified from pENTR/D-FLUC (Guo et al., 2015) with primer PR1-LucF and LucR. The promoter fragment of GhPR1 fused with Luc was cloned into the HindIII/Sacl site of pCambia2301 (Yang et al., 2015) using ClonExpress Ultra One Step Cloning Kit (Vazyme Biotech). The vector pCambia2301-GhPR1pro-Luc was transformed into GV3101, followed by observations and quantitative analysis of the luciferase fluorescence as previously described (Guo et al., 2015).

Yeast one-hybrid assay

Reporter plasmids pHis2.1-GhPR1pro was constructed by inserting the promoter fragment of GhPR1 between EcoRI and Spel sites of pHIS2.1, and pHis2.1-GhPR1pro was used to transform Saccharomyces cerevisiae strain Y187. pGADT7-GhMYB36 was constructed by inserting the entire coding fragment of GhMYB36 between the EcoRI and SacI sites of pGADT7 in a sense orientation, which was also transformed to S. cerevisiae strain Y187. The Yeast One-hybrid Assay was conducted as previously described (Zhang et al., 2018).

Verticillium dahliae inoculations in Arabidopsis and cotton

For V. dahliae inoculations of Arabidopsis, 2–3 weeks old A. thaliana Ecotype Col-0 plants were uprooted, and the roots were immersed for 3 min in a suspension of 10 g conidia per milliliter of V. dahliae culture in Potato Dextrose Broth, Difco™ (Thermo Fisher Scientific, Waltham, MA), following proper washing and rinsing in water. Control plants were treated exactly the same except that their roots were dipped in potato dextrose broth without conidiospores. After replanting in fresh soil, disease development was monitored up to 21 days after inoculation. Verticillium dahliae inoculation of cotton was performed as described previously (Liu et al., 2014b). Quantification of V. dahliae biomass was performed as described previously (Fradin et al., 2011).

RNA-seq analysis of genome-wide gene expression

For RNA-seq analysis, total RNAs were isolated from 2 to 3 weeks old plants of transgenic Arabidopsis overexpressing GhMYB36 and WT using the RNAout kit (Tianze, Beijing, China). The RNA-seq data analysis and the identification of differentially expressed genes (DEGs) were carried out as previously described (de Jonge et al., 2012; Wang et al., 2015). The raw sequencing data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) with accession number PRJNA765119. GO enrichment analysis of DEGs was performed using the bioinformatic tools provided by the Gene Ontology Resources (http://geneontology.org/). GO biological processes were sorted hierarchically by the most specific subclass first, with its parent terms indented directly below it.

Abiotic stress treatments

Cotton plants were grown in soil for to reach three-leaf stage prior to performing abiotic stress treatments. A solution containing 100 μM ABA or 1 μM SA and 0.01% Triton X-100 was sprayed on the cotton leaves for 0, 3, 6, 12, and 24 h of ABA or SA treatment, whereas the spraying with 0.01% Triton X-100 alone served as the Mock treatment.

Western blotting

Protein extractions and immunoblots were performed as previously described (Yu et al., 2012). Total proteins isolated from the transgenic Arabidopsis and cotton plants were assessed using anti-GFP tag primary monoclonal antibody (Sigma-Aldrich, St Louis, MO).
Southern blotting

Cotton transformants were identified by Southern DNA blot analysis as described by Wan et al. (2016). Briefly, approximately 40 µg of genomic DNA from each sample was digested with HindIII overnight. DNA fragments were separated by electrophoresis on a 0.8% agarose gel and were then transferred to a Hybond-N+ nylon membrane (GE Healthcare, Buckinghamshire, UK) and cross-linked by baking at 80°C for 2 h. DIG-dUTP-labeled PCR fragment derived from NPTII gene was used as a probe for hybridization, which was performed by using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Basel, Switzerland) following the manufacturer’s instruction.

Results

GhMYB36 was upregulated by drought stress and ABA

Considering Arabidopsis MYB36 regulates Casparian strip formation and contributes to improve water use efficiencies (Kamiya et al., 2015), it is not unreasonable to assume that its homolog in cotton could also be involved in drought tolerance. To facilitate such an investigation, we have first Blast searched for cotton MYB36 sequences from G. hirsutum protein database (https://www.ncbi.nlm.nih.gov/) using the Arabidopsis MYB36 (AtMYB36) as a query. As a result, 25 proteins were identified as the homologs of AtMYB36 (E value cutoff at 2e-60), four of which were clustered together with AtMYB36 as revealed by phylogenetic analysis (Figure S1); they were NP_001314379.1, XP_016690362.1, and XP_016733400.1, and ASH96785.1 (GhMYB36). GhMYB36 and XP_016733400.1 differ in only one amino acid, that is, 281G in GhMYB36 and 281D in XP_016733400.1, which is considered as a duplicated gene in genome. Under 18% PEG treatment, GhMYB36 showed the highest expression level among these three genes and it was induced at 0.5 h (1.5-fold approximately), and rapidly climbed and it reached the highest level at 48 h (about 6.5-fold; Figure 1a). However, the expression levels of other two genes were about 1/10 that of GhMYB36, highlighting GhMYB36 as a key gene playing an imperative role in response to drought stress.

It was deduced that GhMYB36 possesses two DNA-binding domains (DBD) at its N terminus by SMART protein prediction (http://smart.embl-heidelberg.de/). The C terminus appears to be variable and harbors a stable secondary structure that renders the domain prediction difficult, which is consistent with previous studies on other MYB36 homologs (Pireyre and Burow, 2015). The examination on the spatial expression pattern of GhMYB36 revealed that the highest transcript level was observed in the roots of seedlings, which was about five to ten-fold higher than in stems, true leaves, cotyledons, flowers, and fibers (Figure 1b). Given that ABA may be induced and accumulated as a signal molecule under drought stress, GhMYB36 expression levels was also assessed following ABA treatment. Under 25 mg/L ABA treatment, GhMYB36 expression level was initially induced to approximately 2-fold higher at 3 h, and peaked at 6 h following ABA treatment (about 4-fold; Figure 1c), which demonstrated the inducibility and responsiveness of GhMYB36 by ABA and prompted further investigation on GhMYB36 in response to drought stress.

GhMYB36 was required for drought tolerance in cotton

To investigate the roles of GhMYB36 in drought tolerance, we downregulated the expression of GhMYB36 by using VIGS...
approach (Gu et al., 2014; Zhang et al., 2012). The CLCrV-CHLI vector-treated plants that were used as a positive control exhibited the anticipated photo-bleaching phenotype (Figure S2a). The downregulation of GhMYB36 expression was assessed by qRT-PCR analysis. The expression level of GhMYB36 in the VIGS plants was reduced to 32.7% of the WT, but unaltered in the empty vector (EM) plants (Figure S2b). To examine the possibility of cross-silencing of its paralogs, NP_001314379.1, XP_016690362.1 and XP_016722658.1 that were most closed to GhMYB36 were selected for expression evaluation. The transcript accumulations of these three genes, as revealed by qRT-PCR, were not affected in GhMYB36 VIGS plants (Figure S2b), suggesting the specific down-regulation of GhMYB36 by VIGS. A drought circumstance was then mimicked by submerging the GhMYB36-VIGS plants together with WT plants into 18% PEG for 48 h, and their responses to the osmotic stress were assessed. As shown in Figure 2a, it appeared clear that VIGS down-regulation of GhMYB36 was able to increase plant susceptibility to osmotic stress, as the GhMYB36 VIGS plants exhibited a relatively severe wilting phenotype than both the WT and EM control plants. To quantify the wilting level, the leaf RWC was measured, which was significantly lower in GhMYB36 VIGS plants than in WT and EM plants following PEG treatment, but maintained unaltered normal condition (Figure 2b).

After incubation in 18% PEG for 48 h, the ion leakage in GhMYB36 VIGS plants was significantly higher than that in the WT and EM control plants (Figure 2c), which indicated that the tolerance to osmotic stress by GhMYB36-VIGS cotton plants was substantially compromised.

Malondialdehyde content and T-AOC are two parameters that have been used to assess drought tolerance in plants; a lower MDA content and higher T-AOC than normal imply that the plants are more resistant to drought (Verslues et al., 2006). After incubation in 18% PEG for 48 h, a dramatic increase in MDA content and a significant concurrent reduction in T-AOC were observed in the leaves of GhMYB36 VIGS plants compared to control plants (Figures 2d,e), which corroborated the membrane instability results.

The water loss rate in WT, EM, and VIGS plants was measured as an index for drought tolerance. The first three true leaves of cotton plants under normal growing conditions were detached, and their FW changes were measured over a 10-h period. The VIGS plants showed a faster rate of water loss than the WT and EM plants (Figure S3), suggesting that the expression of GhMYB36 is required for tolerance to drought.

Figure 2  Downregulation of GhMYB36 decreases cotton drought tolerance in cotton. (a) Phenotypes of the cotton seedling treated with 18% PEG. WT, denotes the wild-type cotton cultivar Ao 3503; EM, indicates the EM (CLCrVA)-treated cotton; VIGS, CLCrVA-GhMYB36-treated cotton plants. Photos were taken at 48 h after the stress conditions achieved by the use of 18% PEG. (b) Leaf RWC of WT, EM, and VIGS cotton plants. Values are represented as means ± SE for six plants. Asterisks denote significantly lower values of GhMYB36-silenced cotton plants compared with WT plants (Student’s t-test, **P < 0.01). Experiments were carried out three times. (c) Ion leakage rate in the leaves post 18% PEG treatment for 48 h. The values are expressed as the mean ± SE for the six plants. Asterisks denote significantly higher values of VIGS plants compared with WT plants (Student’s t-test, **P < 0.01). Experiments were carried out three times. (d) MDA content in cotton leaves post 18% PEG treatment for 48 h. The values are expressed as the means ± SE (n = 3) (Student’s t-test, **P < 0.01). (e) T-AOC activities in cotton leaves post 18% PEG treated for 48 h. Values are expressed as the means ± SE (n = 3) (Student’s t-test, **P < 0.01).
Overexpression of \textit{GhMYB36} in Arabidopsis and cotton improved tolerance to drought stress

To confirm the \textit{in vivo} functions of \textit{GhMYB36} during drought stress in plants, \textit{GhMYB36} was ectopically expressed in \textit{A. thaliana}. Sixteen T2 transgenic lines were selected based on kanamycin resistance, and verified by genomic PCR and RT-PCR. Three T3 lines (OE1, OE2, and OE7) that were confirmed by PCR, RT-PCR and western blot (Figure S4) were used to examine drought tolerance. Further, we determined the germination rate of the transgenic seeds on MS medium containing mannitol-mimicking drought stress. There was no significant difference between the transgenic seeds and Col-0 seeds cultured on normal medium (Figures 3a,b), which is in sharp contrast to the transgenic seeds overexpressing \textit{GhMYB36} that showed significantly improved germination rate compared to the Col-0 seeds on the media containing 450 mM or 500 mM mannitol (Figure 3a,b).

Under normal water condition, the \textit{GhMYB36} transgenic seedlings exhibited difference in neither vegetative nor reproductive growth from Col-0 seedlings (Figure 3C, control). However, after 30 d dehydration, only 25\% of the Col-0 plants survived, whereas most of the transgenic seedlings showed normal growth with green and fully expanded rosette leaves (Figures 3c,d). The survival rates of OE1, OE2, and OE7 were approximately 74\%, 71\%, and 74\%, respectively, which was significantly higher than Col-0 (Figures 3c,d). T-AOC activity was also measured to compare the drought tolerance of transgenic Arabidopsis over-expressing \textit{GhMYB36} and WT. Following a period of drought

\begin{figure}[h]
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\caption{Overexpressing \textit{GhMYB36} confers drought tolerance in \textit{Arabidopsis}. (a) Comparison of phenotypes between transgenic lines (OE1, OE2, and OE7) and WT plants under mannitol treatment for 7 days. Bar = 1 cm. (b) Germination rate of transgenic seeds and Col-0 seed under mannitol treatment for 7 days. Asterisks denote significantly higher values of transgenic plants OE1, OE2, and OE7 compared with WT plants (Student’s t-test, \textit{**P} < 0.01). Experiments were carried out three times. (c) Performance of \textit{GhMYB36} transgenic Arabidopsis plants under drought stress. Seedlings were grown without water for 30 days. Bar = 2 cm. (d) Survival rate of Arabidopsis plants under drought stress. Each experiment comprised 20 plants. (Student’s t-test, \textit{**P} < 0.01). (e) T-AOC activities in transgenic Arabidopsis leaves post drought treated for 15 days. Values are expressed as the means ± SE (\textit{n} = 3) (Student’s t-test, \textit{**P} < 0.01).}
\end{figure}

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treatment for 15 days, T-AOC was significantly higher in transgenic Arabidopsis than in WT plants (Figure 3e), which is a manifestation that GhMYB36 confers drought tolerance in Arabidopsis.

The same GhMYB36-overexpressing vector was also transformed into the genome of G. hirsutum by Agrobacterium-mediated transformation. Sixteen T0 plants derived from independent embryogenic calli were generated following kanamycin selection, all of which showed similar phenotypes to WT plants with respect to boll number, and fiber yield and quality (Table S2 and Table S3). The integration of the GhMYB36 construct into cotton genome was confirmed by PCR analysis, RT-PCR, Southern blot, and western blot analyses (Figure S5). The plants of two-weeks old derived from the homozygous T0 transgenic cotton lines OX1 and OX2 overexpressing GhMYB36 were used for drought tolerance analysis when water was withheld for 14 days. The WT lines became severely chlorotic at an early stage, wilted, with many not able to survive. By contrast, OX1 and OX2 showed rather mild wilt symptom and fewer deaths than WT (Figure 4a). After re-watering for 2 days, the survival rate of the transgenic plants OX1 and OX2 was 65% and 75%, respectively, which was substantially higher than WT (<20%) (Figure 4b). Leaf RWC was measured as an important parameter of drought tolerance, for which OX1 and OX2 lines exhibited significantly higher value (>75%) than that of the control plants (about 50%) (Figure 4c).

Consistent with the drought tolerance phenotype, the water loss rate of the detached leaves from both transgenic lines was significantly lower than WT (Figure 4d). Similarly, both OX1 and OX2 exhibited significantly higher T-AOC activity than WT after 24 h of dehydration (Figure 4e). Taken together, these results demonstrated that GhMYB36 positively regulated drought stress response both in Arabidopsis and cotton.

**GhMYB36 upregulated genes relevant to drought tolerance and disease resistance in transgenic Arabidopsis**

To identify the genes that are potentially under the transcriptional regulation by GhMYB36, we performed a RNA-seq analysis of genome-wide gene expression in the seedling of WT and homozygous T2-generation OE1 plants at 21 days after sowing (DAS). Genes with a greater than 4-fold expression change ($\log_2(\text{fold\_change})$) and $P$-value $<0.002$ were considered to be differentially expressed. A total of 82 DEGs that were differentially expressed between WT and OE1 were identified, among which 51 up-regulated and 31 down-regulated in OE1 (Table S4, Figure S6). The DEGs in OE1 revealed significant enrichment for the pathways related to abiotic response, hormone signaling and biotic defense, including the CAP160 protein (AT1G61120.1) response to water deprivation, PR1 (AT2G14610.1) response water deprivation, and defense...
response, and terpenoid synthase 13 (AT4G24000.1) for cell wall organization (Table S4, Figure S6). For validation, ten DEGs were randomly selected in the collected samples to compare their expression by qRT-PCR. As a result, the fold changes in the expression level revealed by qRT-PCR and RNA-seq were closely correlated ($R^2 = 0.91$, $P < 0.05$) (Figure S7). Further, four up-regulated and four down-regulated DEGs in OE1 with relevance to disease and stress resistance were selected for verification by qRT-PCR in transgenic cotton overexpressing GhMYB36 following drought or Verticillium wilt treatments. The four up-regulated DEGs were GH_A10G1631 (LTP4), GH_D12G0420 (PR1), GH_D09G1316 (PR2), and GH_A11G1754 (ABR). The four down-regulated DEGs were GH_A12G2467 (OSM 34), GH_D07G1220 (ROSY1), GH_A08G2482 (peroxidase 44), and GH_A12G2524 (DIR5). All the eight genes showed significant alterations in expression in a consistent manner with the corresponding RNA-seq analysis following drought treatment or Verticillium wilt challenge, with PR1 showing the highest enhancements in expression (Figure S8).

A similar study was conducted to analyze the expression of Casparian strip related genes in Arabidopsis overexpressing GhMYB36. As shown in Table S5, None of these genes showed significant variation in expression, suggesting GhMYB36 may not be a transcriptional regulator for these Casparian strip related genes.

GhMYB36 conferred disease resistance to Verticillium dahliae in Arabidopsis and cotton

To evaluate the potential enhancement in disease resistance under drought stress, 3-week-old seedlings of the chosen three transgenic lines, OE1, OE2, and OE7, and WT control plants were selected and placed under V. dahliae challenge. As WT plants displayed typical V. dahliae infection symptoms, including stunting, wilting, chlorosis, and necrosis, all the transgenic plants were clearly resistant to this fungal disease by showing only minor symptoms (Figure 5a). The phenotypes were apparently correlated with the extent of V. dahliae colonization, which was confirmed by disease indices and qPCR (Figures 5b, c).

Similar observations were made in the two transgenic cotton lines overexpressing GhMYB36, OX1 and OX2 under the treatment with V. dahliae isolate V991 (Figure 5d). Both OX1 and OX2 displayed significantly lower disease indices than WT throughout plant development, and the phenotypes were correlated with the extent of V. dahliae colonization, which was

![Figure 5](image-url)
verified by qPCR (Figures 5e,f). Taken together, these experiments convincingly demonstrated the potential functional role of GhMYB36 in conferring Verticillium resistance in both Arabidopsis and cotton.

GhMYB36 was induced by *Verticillium dahliae* but not by salicylic acid (SA) and was required for *Verticillium dahliae* resistance in cotton.

The potential functionality GhMYB36 in fungal disease resistance was further studied in *V. dahliae* infected cotton roots by qRT-PCR. GhMYB36 expression level was rapidly induced to approximately about 17-fold higher at 4 h post infection and maintained at about 6-fold higher at 20 h post infection (Figure S6a). However, GhMYB36 was not inducible by SA treatment of cotton roots (Figure S6b).

When inoculated with *V. dahliae*, GhMYB36-VIGS cotton plants showed markedly severe disease symptoms including leaf chlorosis and wilting, relative to WT plants (Figure 6a) and an elevated disease index (Figure 6b). The fungal biomass was quantified by using qPCR and the extent of *V. dahliae* colonization was correlated with the phenotypes (Figure 6c). Taken together, these data indicated that GhMYB36 could be an important component in cotton resistance to *V. dahliae* infection.

GhMYB36 is a nucleus localized protein that activates the expression of PR1 gene by binding to the PR1 promoter

To determine the cellular localization of GhMYB36, transient expression of the GhMYB36–GFP fusion protein was conducted by using agro-infiltration in *N. benthamiana* leaves. As shown in Figure 7a, GFP that represents GhMYB36 localization was detected and co-localized with the DNA-specific fluorescent stain DAPI in the nucleus.

As PR1 is well known for its role in conferring disease resistance and drought tolerance (Legrand et al., 1987; Liu et al., 2013; Niderman et al., 1995), its upregulation in Arabidopsis overexpressing GhMYB36 prompted our investigation into the relationship between GhMYB36 and PR1 and test the hypothesis that the GhMYB36-mediated up-regulation of PR1 might contribute to *Verticillium* wilt resistance and drought tolerance by directly regulating PR1 expression in cotton. We employed transactivation assay in *N. benthamiana* using the pPR1::LUC

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**Figure 6** The GhMYB36 gene endowed *Verticillium* wilt resistance in cotton. (a) The phenotypes of Ao3503 under infection by *V. dahliae* isolate V991 after VIGS with CLCrV containing a fragment of GhMYB36 gene. Photos were taken at 30 days after *V. dahliae* inoculation (45 days after VIGS) upper panel, disease symptoms of cotton plants, Bar = 4 cm; middle panel, stem inspection vascular discoloration, Bar = 0.3 cm; bottom panel, recovery assay, Bar = 2 cm. (b) The disease indices GhMYB36 gene-silence lines. The results were presented as means ± SE from three replications with at least 25 plants per replication. (c) Fungal biomass determined by quantitative real-time PCR (R.Q.) in cotton. Bars represent *V. dahliae* ITS transcript levels relative to cotton UBQ14 transcript levels (for equilibration) with SD in a sample of four pooled plants. WT (control) is set to 1. Asterisks indicate significant differences when compared with Col-0 (P < 0.05).
reporter construct to validate the potential binding of whether GhMYB36 directly binds to the promoter region of PR1 and modulate its expression. Firefly luciferase imaging assay showed that 35S-GhMYB36 significantly activated the expression of the Luc reporter gene (Figures 7b,d), indicating that GhMYB36 could directly bind to the promoter of PR1 gene and showed transactivation activity. Such a result was further verified by a yeast one-hybrid assay (Figure 7c).

VIGS down-regulation of GhPR1 under drought treatment or Verticillium challenge was carried out to further examine PR1 functionality. Three weeks after Agrobacterium-mediated VIGS infiltration, qRT-PCR analysis revealed significant reduction in GhPR1 expression (Figure 8b). Consequently, GhPR1 VIGS cotton plants became more susceptible to V. dahliae (Figure 8a) and showed severer wilting phenotype relative to the control plants under drought stress (Figure 8e). Quantitative parameters including disease index, relative fungal biomass, and RWC were consistent with these observations (Figure 8c,d,f).

**Discussion**

MYB TFs have been reported to play a vital role in both abiotic and biotic stresses in plants (Tiwari et al., 2020). As a member of the MYB family, MYB36 has been known to regulate the transition from proliferation to differentiation and to orchestrate Casparian strip formation in Arabidopsis, which suggests its putative function response to abiotic stresses (Kamiya et al., 2015; Liberman et al., 2015). Here, in cotton we discovered that the downregulation of GhMYB36 compromised plants’ tolerance to drought stress and V. dahliae infection (Figures 2 and 6). The opposite was true when GhMYB36 was overexpressed in plants where increased the tolerance to drought stress and V. dahliae resistance were observed (Figures 3 and 5). These results demonstrate a vital functional role that GhMYB36 may have played in response to both abiotic and biotic stresses (Figure 9).
et al., 2014; Pu et al., 2008; Wan et al., 2016; Wang et al., 2004). A few MYB TFs, such as GbMYB5, GaMYB62L, and MYB108, were found to respond to either a fungal challenge or an abiotic stress (Butt et al., 2017; Chen et al., 2015; Cheng et al., 2016), but to our knowledge, our study on GhMYB36 represents the first report of a MYB gene that is involved in both biotic and abiotic stresses in cotton. The dual functional GhMYB36 could be used as a potentially useful tool for genetic manipulation of drought tolerance and disease resistance in cotton, and warrants further studies on its functionality in response to a broad range of biotic and abiotic stresses in both cotton and other plant species, beyond this study on *Verticillium* and drought stress.

The expression pattern of a gene underlying stress conditions has been commonly used to predict its functional role in response to that stress (Gu et al., 2017; Li et al., 2017; Liu et al., 2014a). In this study, the transcript levels of GhMYB36 were raised by the treatments of PEG, ABA, and *V. dahliae*, but not SA as unraveled by qRT-PCR. GhMYB36 was also highly expressed in plant roots. Taken together, these results indicate that GhMYB36 could be involved in the signal transduction of abiotic and biotic stresses. As expected, the overexpression of GhMYB36 led to enhanced tolerance to both drought and *V. dahliae*, indicating that GhMYB36 may act as positive regulator in response to these two stress conditions. The fact that overexpression of GhMYB36 did not give rise to negative impacts on plant growth, renders it great potential for using in genetic engineering to improve drought tolerance and disease resistance.

The downregulation of GhMYB36 in cotton by using VIGS demonstrated that the GhMYB36 VIGS cotton plants were less tolerant to drought and *V. dahliae* compared to WT and EM cotton plants. Earlier research has shown that the changes in the MDA content (Tian et al., 2011) and T-AOC activity of plants affects drought tolerance (Sayfzadeh and Rashidi, 2010). Malondialdehyde that is a product of the peroxidation of unsaturated fatty acids in phospholipids is regarded as an indicator of lipids peroxidation that is responsible for cell membrane damage. The
GhMYB36 confers biotic and abiotic stress resistance

Drought tolerance and V. dahliae resistance in both Arabidopsis and cotton. Although the mechanism of GhMYB36 activities in response to drought and V. dahliae here is attributed to the activation of PR1 expression, possibility exists that GhMYB36 may target additional genes to fulfill its potentially diverse functions. This report has not only elucidated the molecular mechanism underlying the functional role of GhMYB36 in response to both biotic and abiotic stresses, but also provided a useful tool for molecular breeding and biotechnological advance for improving crop production.

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Author contributions

TL and TC contributed equally to this work. TL, TC, DG, and BZ designed the study. TL, TC, JK, YY, XL, and LX performed the research. TL, JK, JW, and YY analyzed the data. The data were discussed by TL and BZ who also wrote the manuscript.

Conflict of interest

No conflicts of interest declared.

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Figure 9 A model for GhMYB36 in cotton responses to biotic stress and abiotic stress. Arrows indicate positive regulation. Solid and dashed arrows indicate direct action and predicted indirect action respectively.

Mechanisms for detoxification of active oxygen species exist in all plants, which include the activation of a number of enzymes, such as superoxide dismutase, catalase, ascorbate peroxidase, peroxidase, and glutathione reductase (Sayfzadeh and Rashidi, 2010; Ullah et al., 2017). Plants have evolved antioxidant defense systems to scavenge excess reactive oxygen species (ROS) and maintain cellular ROS homeostasis. Therefore, MDA content and T-AOC activity were measured in both the GhMYB36-VIGS cotton plants and those overexpressing GhMYB36. Under stress treatment, the former exhibited greater MDA content and lower T-AOC activities than WT and EM control plants, whereas the opposite was true for the latter that exhibited reduction in MDA content and enhancement in T-AOC activities. It is likely that the higher expression of GhMYB36 promoted antioxidant enzyme activities that are responsible for maintaining membrane integrity and intracellular osmotic pressure of cell under drought stress, as the functional role of MYB family in ROS scavenging through enhanced antioxidant activity has been very well characterized in stress management (Qin et al., 2012).

The PR gene family has been widely used as molecular markers in plant defense responses (Liu et al., 2013; Mitsuhara et al., 2008). Most PR genes are expressed at a basal level under normal growth conditions, but can be rapidly induced by pathogenic infections (Liu et al., 2013; Mitsuhara et al., 2008). To a lesser extent, they were also found in response to abiotic environmental stimuli (Liu et al., 2013; Seo et al., 2008). For example, the expression of Arabidopsis PR1 was induced by drought or salt stress and its overexpression led to enhanced tolerance to drought stress relative to WT plants (Seo et al., 2008). In tomato (Solanum lycopersicum), drought stress resulted in upregulation of all SIPR-1 genes up to 50 folds higher than those at normal condition (Akbudak et al., 2020). In this study for the first time, we have unraveled the molecular mechanisms underlying the functional role of GhMYB36 in conferring tolerance to drought and Verticillium resistance, through the activation of PR1 by direct binding of GhMYB36 on its promoter. The observation that GhMYB36 did not regulate the Casparian strip genes manifested its peculiar functional pathway that is distinct from other plant species (Kamiya et al., 2015; Liberman et al., 2015). In summary, GhMYB36 functions as a transcription factor that improves the
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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Phylogenetic tree of Arabidopsis thaliana MYB36 related protein in Gossypium hirsutum.

Figure S2 Virus-induced gene silencing of GhMYB36 in cotton.

Figure S3 Virus-induced gene silencing of GhMYB36 enhanced the water loss rate in the leaves.

Figure S4 Detection of GhMYB36 positive transgenic Arabidopsis lines via PCR, RT-PCR and western blot.

Figure S5 Detection of two TO GhMYB36 transgenic cotton lines via PCR, RT-PCR, western blot, and Southern blot.

Figure S6 GO enrichment analysis of DEGs.

Figure S7 Verification of the expression patterns of differentially expressed genes using quantitative RT-PCR.

Figure S8 Transcriptional fold changes of eight genes selected from DEGs of Arabidopsis RNA-seq data after drought or Verticillium wilt treatment in cotton

Figure S9 GhMYB36 was induced expression by V. dahliae but not SA.

Table S1 Oligonucleotides used in the study.

Table S2 Cotton yield and boll number of wild type and two independent transgenic cotton lines overexpressing GhMYB36 under normal condition.

Table S3 Fiber quality under normal condition.

Table S4 The differentially expressed genes between WT and Arabidopsis overexpressing GhMYB36.

Table S5 The Casparian strip related genes expression data between WT and Arabidopsis overexpressing GhMYB36.