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Original Article

Overexpression of cotton (*Gossypium hirsutum*) *dirigent1* gene enhances lignification that blocks the spread of *Verticillium dahliae*

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Dirigent super-family abounds throughout the plant kingdom, especially vascular plants. To elucidate the function of cotton (*Gossypium hirsutum*) *DIR* genes in lignification, two cDNAs (designated *GhDIR1* and *GhDIR2)* encoding putative dirigent proteins were isolated from cotton cDNA libraries. Real-time quantitative reverse transcription-polymerase chain reaction analysis revealed that *GhDIR1* transcript was preferentially accumulated in cotton hypocotyls, whereas *GhDIR2* was predominantly expressed in cotton fibers. Overexpression of *GhDIR1* gene resulted in an increase in lignin content in transgenic cotton plants, compared with that of wild type. Histochemical assay revealed that the transgenic plants displayed more widespread lignification than that of wild type in epidermis and vascular bundle. Furthermore, the transgenic cotton plants displayed more tolerance to the infection of *Verticillium dahliae*. Our data suggest that *GhDIR1* may be involved in cotton lignification which can block the spread of fungal pathogen *V. dahliae*.

Keywords cotton (*Gossypium hirsutum*); dirigent protein; lignin; *Verticillium* wilt; overexpression

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Introduction

Lignin is an essential constituent of the cell walls of all vascular plants. It is a complex, phenolic-based polymer composed of phenylpropanoid units derived from three monolignols: sinapyl, E-coniferyl, and p-coumaryl alcohols [1]. In dicotyledonous plants, sinapyl and E-coniferyl alcohol monolignols are biosynthesized in cytoplasm [2]. The lignin monomers are subsequently transported to the cell wall where they are polymerized in a combinatorial fashion by free-radical coupling mechanisms [3] or under the control of dirigent proteins [4], generating a variety of structures within the lignin polymer. By using phloroglucinol–HCl histochemical staining, patterns of lignin deposition are readily visualized within the stem xylem cell walls and phloem fiber cells. It is well known that lignin provides structural support, enables water transport, and contributes to plant defense mechanisms against both biotic and abiotic stresses [5].

The first *DIR* (*dirigent*) gene regulating the coupling of two monolignol radicals to yield lignans and lignins was discovered in *Forsythia intermedia* [6,7]. However, only E-coniferyl alcohol, but not p-coumaryl or sinapyl alcohols that differ only in the degree of methoxylation, was guided by DIR. Additionally, with the formation of radicals, oxidative enzymes (such as peroxidase or laccase) are required for the dehydrogenation of the monolignols [8]. Moreover, a family of nine *DIR* genes of western red cedar (*Thuja plicata*) was also shown to function in regulating E-coniferyl alcohol coupling to produce (+)-pinoresinol [9]. Poplar (*Populus s*pp.) *DIR* gene is highly expressed in the lignifying zone [10], supporting an important role for *DIR* genes in these tissues. Burlat *et al.* [11] revealed that the DIR protein has been immunolocalized to the cambial region and the cell wall. Several weeks after inoculation of Norway spruce bark and cambium with the bark beetle-associated fungal pathogen *Ceratocystis polonica*, cells surrounding the inoculation site become partially or completely lignified [12,13]. This lignification likely attributes to strengthening cell walls to block the spread of the fungal pathogen, possibly via induction of DIR activity [14].

DIR and *DIR*-like super-families abound in most of plant species. Up to date, 25 *DIR* members in *Arabidopsis*, 30 members in rice (*Oryza sativa*), 19 members in spruce, and 3 members from *F. intermedia* have been identified [7,14]. *DIR* genes have also been characterized in loblolly pine (*Pinus taeda*), western red cedar (*T. plicata*), western

hemlock (*Tsuga heterophylla*), eucommia (*Eucommia ulmoides*), Manchurian ash (*Fraxinus mandschurica*), quaking aspen (*Populus tremuloides*), sesame (*Sesamum indicum*), *Schisandra chinensis*, creosote bush (*Larrea tridentata*), *Piper futokadsura*, flax (*Linum usitatissimum*), tobacco (*Nicotiana tabacum*) [4], and *Gossypium barbadense* [15]. To our knowledge, little information is available on cotton *DIR* genes, including their expression and function in cotton.

Cotton (*Gossypium* spp.) is an economically important crop in the world. In the last few years, some efforts have been made to improve cotton quality and yield through identifying the potential cotton genes for genetic manipulation [16–21]. More recently, two studies reported that the enhanced plant resistance to fungal pathogen *Verticillium dahliae* has been achieved by the overexpression of cotton genes in transgenic plants [22,23]. Here, we described molecular characterization of two cotton *DIR* genes (designated *GhDIR1* and *GhDIR2*) encoding putative dirigent proteins with a typical dirigent domain. Overexpression of *GhDIR1* in cotton enhanced lignin content and delayed the spread of fungal pathogen *V. dahliae* in transgenic plants.

Materials and Methods

Plant materials

Cotton (*Gossypium hirsutum* cv Xuzhou142 and Coker312) seeds (preserved in our lab) were surface-sterilized with 70% (v/v) ethanol for 1 min and 10% (v/v) H_2O_2 for 2 h, followed by washing with sterile water for 3–5 times. The sterilized seeds were then germinated on half-strength Murashige and Skoog (MS) medium (pH 5.8) containing 0.7% agar under a 16-h-light/8-h-dark cycle at 28–30°C. Roots, cotyledons, and hypocotyls were collected from 2-week-old sterile seedlings of cotton cv. Xuzhou 142. Other tissues (including young leaves and stems from 4-week-old seedlings, petals and anthers from the non-flowering plants, and ovules and fibers from plants after anthesis) were collected from cotton plants grown in a greenhouse.

Isolation of *GhDIR* cDNAs

To identify the genes involved in lignin biosynthesis, over 10,000 cDNA clones were randomly selected from cotton cDNA libraries constructed earlier [24] for sequencing. Two *GhDIR* cDNA clones were obtained through sequence analysis.

DNA and protein sequence analysis

The open reading frame (ORF) of *GhDIR* genes and their deduced protein sequences were analyzed using DNASTAR software (DNAStar Co., Madison, USA). The conserved domain was determined by NCBI Conserved Domain Search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment of GhDIR with other plant DIRs were performed using ClustalW (http://www.ebi.ac.uk/clustalw/) and protein motif analysis using motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). N-glycosylation of the putative cotton DIR proteins were investigated using NETNGLYC (http://www.cbs.dtu.dk/services/NetNGlyc/). The evolutionary relationship of the DIR proteins was determined by MEGA3.1 software (http://www.megasoftware.net/), based on minimum evolution from 1000 bootstrap replicates. The signal peptides of deduced protein sequences were analyzed using SignalP (http://www.cbs.dtu.dk/services/SignalP).

Reverse transcription-polymerase chain reaction analysis of *GhDIR* expression

Expression of the *GhDIR* genes in cotton tissues was analyzed by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) with the fluorescent intercalating dye SYBR-Green in a detection system (Opticon 2; MJ Research,Waltham, USA). A cotton polyubiquitin gene (*GhUBI1*) was used as a standard control in the RT-PCR reactions [17]. A two-step RT-PCR procedure was performed in the experiments. First, 2 μg of purified total RNA was reversely transcribed into cDNAs by M-MLV reverse transcriptase. The cDNAs were used as templates for RT-PCR reactions using gene-specific primers (*GhDIR1* P1: 5′-AGGCTATACTGGCACGACATT-3′; P2: 5′-CCCACAACTCCATATTTTAATAAC-3′. *GhDIR2* P1: 5′- AAAGCTAAGTCACTTTCGCATCTA-3′; P2: 5′-TGGGGCAGTACAATATGATTAAAG-3′). Real-time PCR reaction was performed using PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Relative quantification of *GhDIR* expression was determined using the comparative Ct method [17]. To achieve optimal amplification, PCR conditions for each primer combination were optimized for annealing temperature, and PCR products were verified by melting curve analysis and confirmed on an agarose gel. Mean values and standard errors (bar) were calculated from three independent experiments with three biological replicates of cotton materials, and the data were normalized with the relative efficiency of each primer pair.

Construction of the *CaMV35S:GhDIR1* overexpression vector and cotton transformation

To construct *GhDIR1* overexpression vector, the ORF of *GhDIR1* gene was amplified by PCR using *Pfu* DNA polymerase with gene-specific primers and inserted subsequently into a pBluescript II SK+ vector at the sites *Bam*HI and *Sac*I, creating an intermediate construct (pSK-*GhDIR1*). The pSK-*GhDIR1* construct was digested with *Bam*HI and *Sac*I, and then the *GhDIR1* ORF fragment was cloned into

pBI121 vector at *Bam*HI/*Sac*I sites, replacing the *GUS* gene (named pBI121-GhDIR1).

Hypocotyl explants of cotton (Coker 312) sterile seedlings were transformed with the pBI121-GhDIR1 construct, using *Agrobacterium tumefaciens*-mediated DNA transfer as described previously [24]. Homozygosity of transgenic plants was determined by segregation ratio of the kanamycin selection marker and further confirmed by PCR analysis. The transgenic cotton seedlings (T0–T2) were selected on half-strength MS medium containing 25 μg/ml kanamycin and transplanted the seedlings into pots.

Verticillium dahliae infection of cotton plants

Verticillium dahliae (strain Bima90–15) was grown on potato dextrose agar plat at room temperature (25°C) for 7–10 days and then was inoculated into Czapek broth on a shaker at 110 rpm at 25°C for 10 days until the concentration of spores reached $1 \times 10^8 - 1 \times 10^9$ conidia/ml. The spore suspensions were adjusted to $0.94 \times 10^7 - 1 \times 10^7$ conidia/ml with sterile distilled water for inoculation [25], and root-inoculated into both control and transgenic plants using a syringe needle at the bottom pots when the first leaf appeared. Control plants were not inoculated but were otherwise treated and sampled with distilled water in the same way. The infected seedlings were examined for symptoms or used for further experiments after 2–3 months.

Analysis of cell wall composition

The hypocotyls of 2-week-old transgenic and wild-type cotton plants were ground into fine powder in liquid nitrogen. The fine powders were washed with 70% aqueous ethanol and pelleted by centrifugation at 10,000 *g* for 15 min. The resulting pellets (cell wall samples) were washed with a mixture of chloroform and methanol (1 : 1, v/v) and then washed twice with acetone before freeze drying.

A total of 0.2 g cell wall (alcohol-insoluble material, AIR) sample was hydrolyzed in 4 ml of 72% H_2SO_4 at 30°C for 2 h. The hydrolysates were diluted by adding 112 ml of H_2O and then autoclaved at 121°C for 2 h. The solution was filtered through a fritted glass crucible. The samples were washed using hot water and dried. Lignin content was measured and expressed as a percentage of total dry weight (the original weight of cell wall residues) [26].

Ten milligrams of total cell wall materials was subjected to 2 M trifluoroacetic acid at 120°C for 2 h to produce monosaccharides. The neutral monosaccharides were converted into alditol acrates whereas uronic acids were derivatized by trimethylsilyl methoxime before gas chromatography/mass spectrometry (GC/MS) analysis on Agilent GC6890N [27].

Histochemical assay of lignin

Histochemical examination of lignin in the cotton stems was carried out using phloroglucinol [28]. The hypocotyls at ~10 mm above roots of the transgenic cotton plants were bare-handedly cut into ~0.5 mm thin slices, using wild-type hypocotyls as controls ($n > 50$ slices for each transgenic line and wild type). Cross-sections were incubated for 10 min in a phloroglucinol solution (2% in 95% ethanol), treated with 18% HCl for 5 min, and directly observed with a bright-field microscope (Leica DMR, Wetzlar, Germany). The experiments were repeated at least three times.

Results

Isolation of *GhDIR* cDNAs

To identify genes that might be involved in lignin biosynthesis, 10,000 cDNA clones from cotton cDNA libraries were randomly sequenced. Among them, two clones (designated *GhDIR1* and *GhDIR2*, accession number in GenBank: FJ600364 and FJ600365) were identified to encode putative dirigent proteins. *GhDIR1* and *GhDIR2* genes containing 573 and 594 bp of ORFs encode the proteins with 190 and 197 amino acids, respectively. The ORF of *GhDIR1* shares 59.5% identity with that of *GhDIR2* gene.

Phylogenetic relationship of GhDIRs with other plant DIR proteins

To investigate the evolutionary relationship of the cotton dirigent proteins with other dirigent proteins, all of the known cotton DIRs, poplar DIRs, and *Arabidopsis* DIRs were selected from GenBank for phylogenetic analysis. As shown in **Fig. 1**, the dirigent proteins can be divided into two subgroups. GhDIR1 and GhDIR2 are classified into the first subgroup of the tree, while GbDIR1 and GbDIR2 are in the second subgroup. GhDIR1 shares much closer evolutional relationship with AtDIR23 (BT005788). GhDIR2 is relatively closer to PtDIR7 (XP_002323338) and AtDIR18 (AY081267). According to Ralph's classification [29], GhDIR1, GhDIR2, and most of PtDIRs were classified to DIR-e group, while GbDIR1 and GbDIR2 to DIR-b/d group. These results suggest that DIR proteins exhibit much diversity in phylogeny even between different cotton species.

Structural analysis of the GhDIR proteins

Predicted GhDIR proteins consist of a typical DIR domain and several other conserved motifs (i.e. N-glycosylation site, N-myristoylation site, and protein kinase C phosphorylation site). As shown in **Fig. 2**, the N-terminus of GhDIR1 contains a putative signal peptide (SP) (M_1–A_{24}). The DIR domain (V_{20}–H_{189}) contains some important motifs, such as four N-glycosylation sites (PGSs; N in NxS/T motifs, position 26, 61, 73, and 130), some putative N-myristoylation sites (G_{27}SSFAR$_{32}$, G_{58}GKNAT$_{63}$,

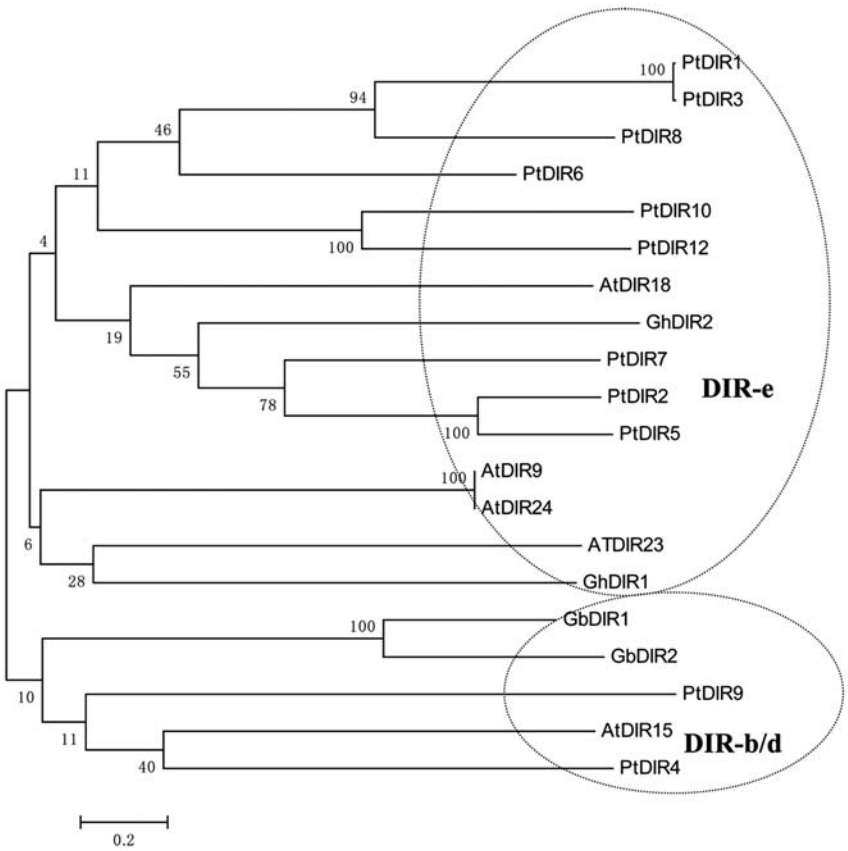

Figure 1 Phylogenetic relationship of cotton dirigent proteins to other dirigent proteins The accession numbers of plant dirigent proteins in GenBank are: AtDIR9 (BT010722), AtDIR15 (AEE86966), AtDIR18 (AY081267), AtDIR23 (BT005788), AtDIR24 (AEC09674), GhDIR1 (ACU55135), GhDIR2 (ACU55136), GbDIR1 (AAS73001), GbDIR2 (AAY44415), PtDIR1 (XP_002335799), PtDIR2 (XP_002297648), PtDIR3 (XP_002304013), PtDIR4 (ABK94138), PtDIR5 (XP_002336701), PtDIR6 (ABK93789), PtDIR7 (XP_002323338), PtDIR8 (XP_002323336), PtDIR9 (XP_002323337), PtDIR10 (XP_002313728), and PtDIR12 (XP_002297761). At, *Arabidopsis thaliana*; Gh, *Gossypium hirsutum*; Gb, *Gossypium barbadense*; Pt, *Populus trichocarpa*.

G_{115}LLMAM$_{120}$, G_{131}STITI$_{136}$), and putative protein kinase C phosphorylation sites (T_{89}MR$_{91}$, S_{95}SK$_{97}$), which are possibly important for the activity of GhDIR1. In the DIR domain (K_{49}–H_{196}) of GhDIR2, there are five N-glycosylation sites (PGSs; N in NxS/T motifs, position 68, 79, 100, 137, and 180) and three putative protein kinase C phosphorylation sites (S_{65}GR$_{67}$, S_{71}IR$_{73}$, S_{102}SK$_{104}$). Sequence alignment analysis revealed that GhDIR1 shares the highest similarity (70% identity) with PtDIR4 (ABK94138), and relatively high homology (62% identity) with GhDIR2. It also shares 60% identity with PtDIR1 (XP_002335799) and PtDIR2 (XP_002297648), but relatively low homology with other known plant DIRs.

Expression analysis of *GhDIR1* and *GhDIR2* in cotton tissues

For characterization of *GhDIR* expression patterns in cotton, quantitative RT-PCR analysis was performed. As shown in **Fig 3**, *GhDIR1* is preferentially expressed in cotton hypocotyls. The transcripts of *GhDIR1* were also detected in roots, cotyledons, and ovules. Very low levels of *GhDIR1* expression were found in leaves, stems, anthers, and fibers. In contrast, *GhDIR2* is highly expressed in cotton fibers, but barely detectable in roots, cotyledons, leaves, hypocotyls, stems, petals, anthers, and ovules. Moreover, time-course analysis revealed that *GhDIR2* transcripts were detected at a relatively low level in fibers at 2 days post-anthesis (DPA), and its expression level reached the highest peak in 5 DPA fibers with a relative value of 655.5. Afterwards, it declined gradually to 286 in 10 DPA fibers, and 8.78 in 15 DPA fibers (**Fig. 3**), suggesting that the expression of *GhDIR2* is regulated during fiber development of cotton. To investigate the role of cotton *DIR* gene in lignin biosynthesis, we chose the *GhDIR1* gene for further functional analysis, as the cell wall of cotton fiber contains little lignin.

Overexpression of *GhDIR1* gene increases lignin content of cotton plants

To investigate the phenotypic effects of *GhDIR1* gain of function, we generated more than 30 transgenic cotton plants overexpressing *GhDIR1* gene under the control of

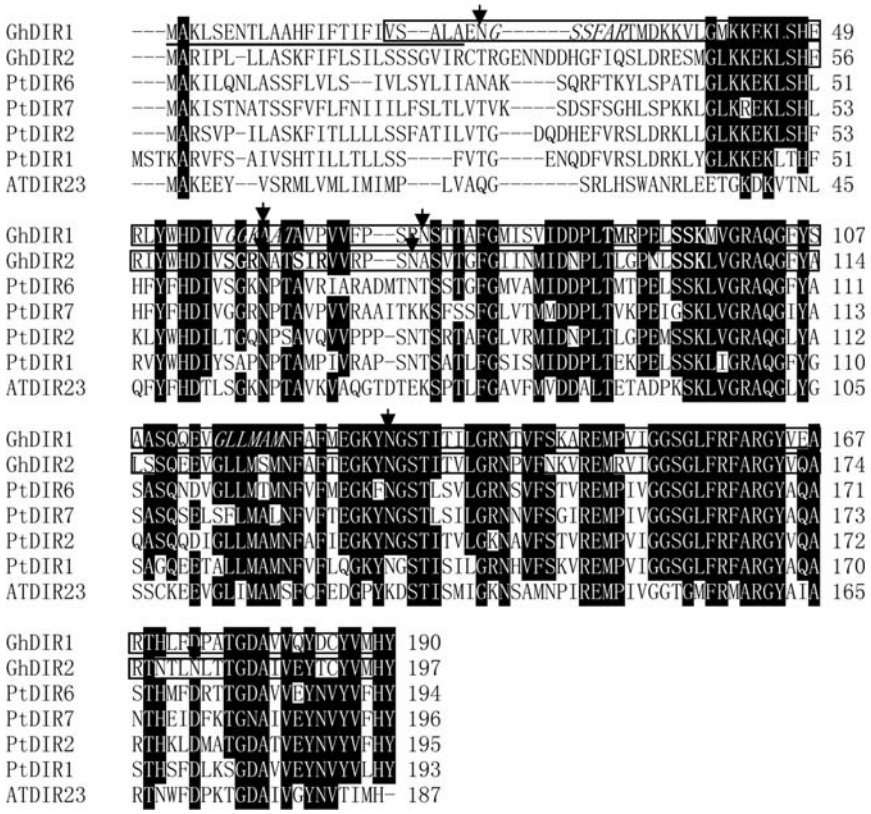

Figure 2 Multiple alignment of deduced amino acid sequences of plant DIR proteins Amino acid sequences were aligned using ClustalW software. Black shading shows amino acid identities, the putative signal peptide is underlined, arrows show putative N-glycosylation sites, the putative N-myristoylation sites are italic, the putative phosphorylation sites are in bold, and black boxes represent DIR domain.

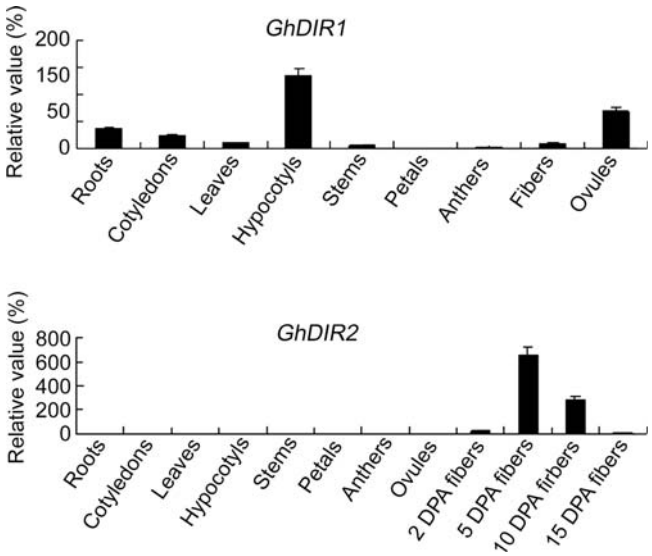

Figure 3 Quantitative RT-PCR analysis of *GhDIR1* and *GhDIR2* genes expression in cotton tissues Total RNA was isolated from cotton tissues, and relative value of *GhDIR* expression in cotton tissues was shown as percentage of *GhUBI1* expression activity. Mean values and standard errors (bar) were shown from three independent experiments.

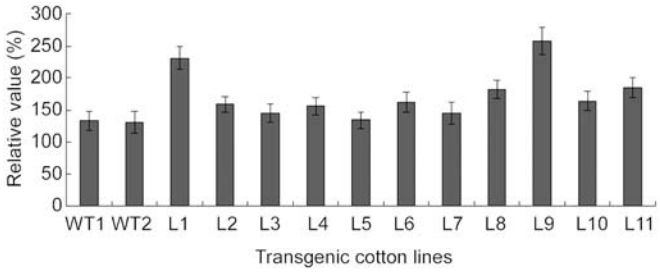

Figure 4 Quantitative RT-PCR analysis of *GhDIR1* gene expression in hypocotyls of the transgenic cotton plants Total RNA was isolated from 2-week-old hypocotyls of transgenic cotton seedlings and wild type. Relative value of *GhDIR1* expression in cotton hypocotyls was shown as a percentage of *GhUBI1* expression activity. Mean values and standard errors (bar) were shown from three independent experiments. WT1 and WT2, wild type; L1–L11, transgenic lines.

the constitutive CaMV 35S promoter. To demonstrate the increasing *GhDIR1* expression in the transgenic plants, we analyzed *GhDIR1* expression in the transgenic seedlings by quantitative RT-PCR. Our data indicated that the expression levels of *GhDIR1* in some transgenic cotton progenies (T1–T4 generations) were remarkably enhanced, compared with those of non-transgenic plants (wild type) (**Fig. 4**). As the expression of *GhDIR1* gene may be correlated with lignin polymerization, lignin content and soluble sugar composition of cell walls were quantitatively measured in the hypocotyls and stems of those transgenic cotton lines with the increased *GhDIR1* expression. We chose two transgenic lines (L1 and L9) with the highest levels of *GhDIR1* expression (relative value of 231.5 and

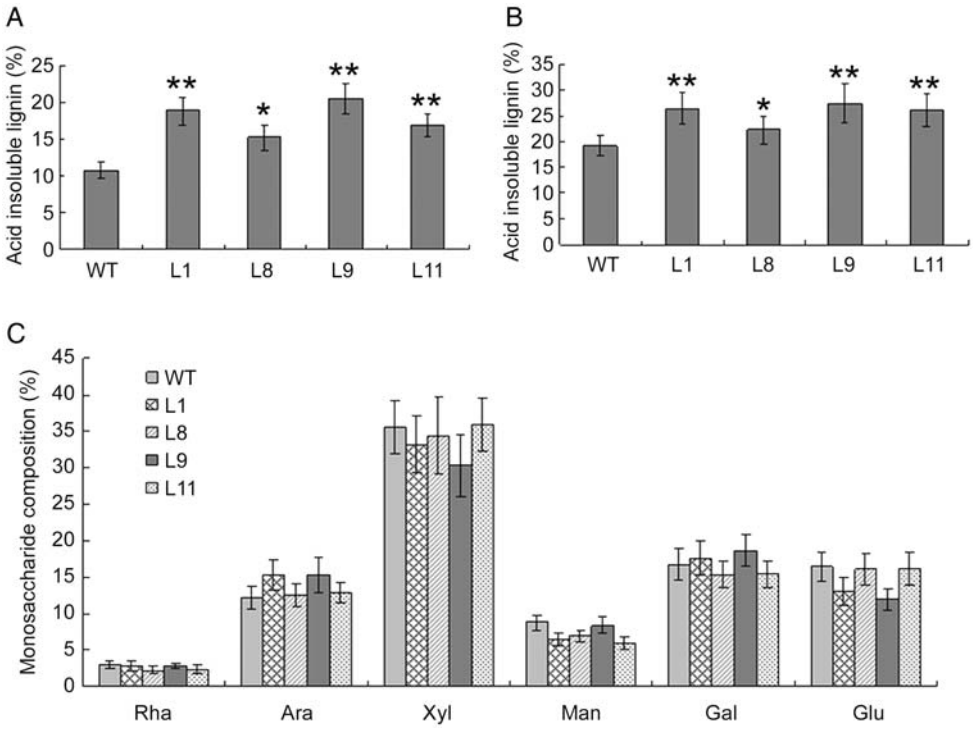

Figure 5 Analysis of lignin content and non-cellulosic neutral monosaccharide composition of cell walls in transgenic cotton plants (A) Lignin content in hypocotyls. (B) Lignin content in stems. (C) Non-cellulosic neutral monosaccharide composition analysis of the cell walls. $*P < 0.05$, $**P < 0.01$ compared with wild-type control by *t*-test. Error bars were calculated from three independent experiments. Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glu, glucose; WT, wild type; L1, L8, L9 and L11, transgenic cotton lines 1, 8, 9 and 11.

256.4, respectively) and two lines (L8 and L11) with relative higher expression (relative value of 182.1 and 184.8, respectively) for further assaying the lignin content and sugar composition, using the wild type (its relative value of 130.3–132.6) as control. The experimental results indicated that lignin content in hypocotyls of the transgenic cotton lines was much higher than that of wild type [**Fig. 5(A)**]. The lignin content in transgenic plants (L9) was ~2 folds higher than that of the control. Likewise, lignin content in stems of the transgenic cotton plants was also significantly enhanced [**Fig. 5(B)**]. In addition, we analyzed the sugar composition of alcohol-insoluble material (AIR) of cell walls by GC/MS. However, there was little difference between wild type and transgenic lines [**Fig. 5(C)**], indicating that overexpression of *GhDIR1* gene may not alter the other components of the cell walls. As *GhDIR1* was preferentially expressed in cotton hypocotyls, we detected the lignin in cross-sections of hypocotyls by the phloroglucinol-HCl (Wiesner) reaction. At least 10 hypocotyls (i.e. 50–60 cross sections) of each transgenic line and the same number of wild type were used in each experiment. Histochemical assay revealed that the transgenic plants displayed more widespread lignification than that of wild type in 2-week-old hypocotyl vascular bundles [**Fig. 6(A–H)**]. We analyzed the pink-stained areas in total section and found that the ratio of transgenic lines was significantly higher than wild type [**Fig. 6(I)**]. These results suggest that GhDIR1 may be involved in cotton lignification.

Overexpression of *GhDIR1* gene in cotton delays invasion of fungal pathogen *V. dahliae*

To investigate whether overexpression of *GhDIR1* gene enhances cotton disease resistance, the transgenic cotton plants were infected with fungal pathogen *V. dahliae*, using the non-transgenic cotton plants (wild type) as controls. As shown in **Fig. 7(A)**, the transgenic and non-transgenic seedlings were transplanted in a same pot (at least 30 pots in each test). One month later, cotyledons of the wild-type plants began to manifest necrotic lesion caused by *V. dahliae*, whereas there was no or a few necrotic lesion on those of the transgenic cottons. Until about one-and-a-half months, similar symptom was observed on the cotyledons of the transgenic *GhDIR1* cotton plants, demonstrating that the transgenic cottons are at least 2-week delay in the fungal invasion, compared with the wild-type control. Necrotic lesion on the leaves of cotton plants was also investigated. At ~2 months post-infection, the leaves of the wild-type plants were gradually dying off, whereas the leaves of the transgenic cottons only began to manifest necrotic lesion [**Fig. 7(B)**]. At this time, the initial symptom on cotton plants is usually an epinasty of the

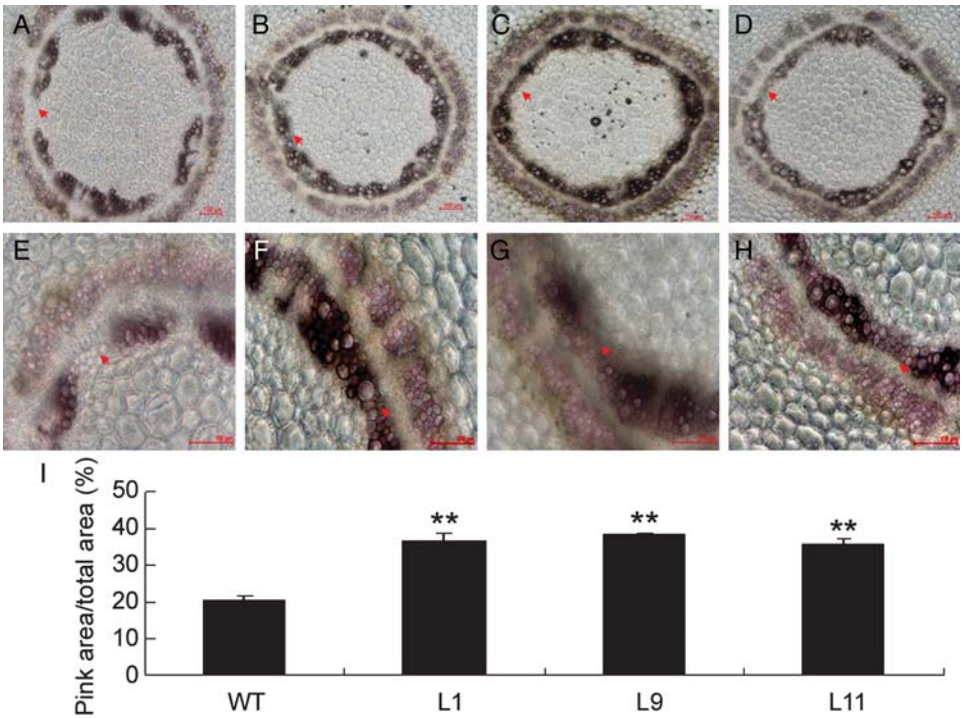

Figure 6 Histochemical assay of lignin in hypocotyls of transgenic cotton plants with overexpressing *GhDIR1* (A,E) A cross-section of the wild-type hypocotyls. (B,F) A cross-section of hypocotyls of the transgenic cotton line 1 (L1). (C,G) A cross-section of hypocotyls of the transgenic cotton line 9 (L9). (D,H) A cross-section of hypocotyls of the transgenic cotton line 11 (L11). (I) Statistical analysis of pink-stained area in total section area. $^{**}P < 0.01$ compared with wild-type control by *t*-test. At least 10 hypocotyls (i.e. $n > 50$ cross sections) of each transgenic line and the same number of wild type were used in each experiment. Cross sections of hypocotyls of transgenic cotton seedlings (including lines L1, L9, and L11) were stained with Wiesner reagents, using non-transgenic plants (wild type) as controls. The photographs were captured under Leica DM4000B microscope. Scale bar = 100 μm.

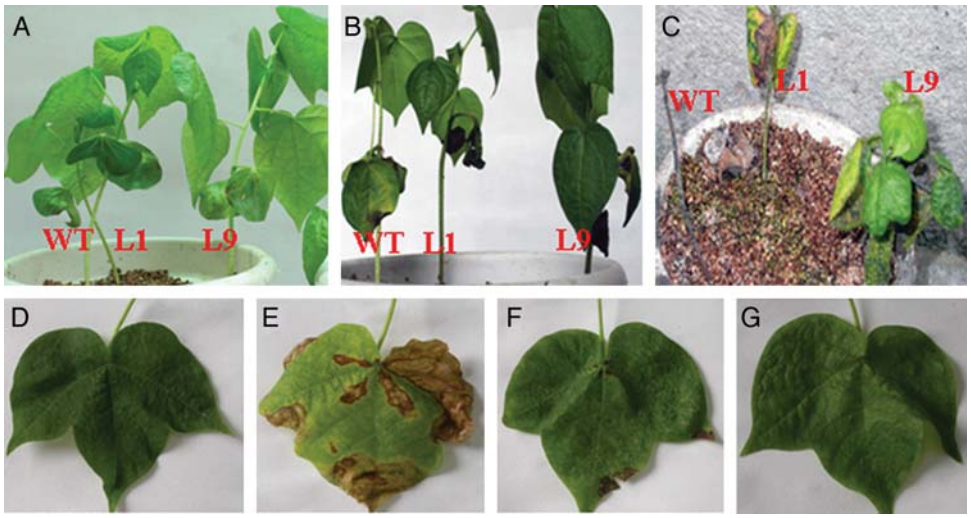

Figure 7 Symptom of transgenic cotton plants infected with *V. dahliae* (A) Non-infected cotton seedlings. (B) Cotton seedlings infected with fungal pathogen *V. dahliae* for 2 months. (C) Cotton seedlings infected with fungal pathogen *V. dahliae* for 3 months. (D) A wild-type leaf not infected with *V. dahliae*. (E) A wild-type leaf infected with *V. dahliae* for 2 months. (F, G) Leaves of transgenic lines L1 (F) and L9 (G) infected with *V. dahliae* for 2 months. The third leaves were collected from the top of cotton plants ($n = 30$ for each trial).

terminal leaves. Further development of symptoms included chlorosis, necrosis, abscission of leaves, and plant stunting. To investigate the incidence of necrotic lesion on the leaves of cottons, the third leaves from the top of non-transgenic and transgenic cotton plants were collected in the same infection time [**Fig. 7(D–G)**]. The necrotic lesion spread all over the wild-type leaves [**Fig. 7(E)**], and this symptom represented on ~85% non-transgenic cotton

plants. On the contrary, the leaves of the transgenic cotton plants displayed a few necrotic lesions [**Fig. 7(F,G)**]. Only two or three necrotic lesions were observed on the leaves of >62% plants of the transgenic line L1. Interestingly, the transgenic cotton L9 plants were more tolerant to the fungal infection [**Fig. 7(G)**]. No necrotic lesion was observed on the leaves of ~30% plants of the transgenic line L9 after the fungal infection for 2 months. At 3 months post-infection, non-transgenic cotton plants infected with Verticillium wilt died with severely necrotic leaves, whereas a portion of the transgenic plants still survived from the infection of *V. dahliae* [**Fig. 7(C)**]. Statistical analysis indicated that there were significant differences ($P < 0.05$ in *t*-test) in the infection between the transgenic lines and controls (**Supplementary Table S1**). These results suggest that overexpression of *GhDIR1* gene in cotton significantly delays the invasion of fungal pathogen *V. dahliae*.

Discussion

A large number of *DIR* gene homologues have been described in various plant species [4]. In moco cotton, a dirigent protein mediates stereoselective coupling of hemigossypol to form (+)-gossypol [30]. In *Forsythia suspense*, the corresponding native subunit of a dirigent protein was found to be glycosylated with a subunit size of ~26–27 and 21–23 kDa as determined by SDS-PAGE [31] and MALDI-TOF [4], respectively. A *F. intermedia* gene was cloned and its encoded dirigent protein had the fully functional glycosylated recombinant form [7]. Likewise, the proteins deduced from the isolated cotton *DIR1* and *DIR2* genes contain typical dirigent (DIR) domain and N-glycosylation sites, suggesting that they belong to DIR super-family.

The members of *DIR* super-family are differentially expressed in plant tissues. A *DIR* gene, *psd-Fi1*, was expressed in *F. intermedia* stem, root, and petiole tissues, detected by tissue print mRNA hybridization [32–34] and in situ hybridization [35]. Moreover, gross localization of the *DIR* mRNA was mainly associated with the cambial regions [36]. Similarly, our results reveal that *GhDIR1* is preferentially expressed in cotton hypocotyls, whereas *GhDIR2* is specifically expressed in cotton fibers.

Verticillium dahliae is a phytopathogenic fungus that causes *Verticillium* wilt vascular disease in many crops, including cotton. This disease is widespread in most cotton-growing areas and is a major threat to cotton production. Cotton plants infected with *V. dahliae* manifest a number of different symptoms. To control this serious disease, a few potential disease-resistant genes have been identified in cotton in recent years. A previous study showed that expression of *GhHb1* gene, a cotton non-symbiotic hemoglobin gene, is activated in cotton roots after *V. dahliae* infection [37]. Further study revealed that ectopic overproduction of the *GhHb1* gene in *Arabidopsis* enhances plant tolerance to *V. dahliae*, suggesting that the GhHb1 protein is involved in defense against pathogen invasions [38]. Genetic transformation of cotton with a harpin-encoding gene *hpaXoo* from *X. oryzae pv. Oryzae* confers an enhanced defense against *V. dahliae* [39]. Recently, a study revealed that a sea-island cotton (*G. barbadense*) thaumatin-like gene (*GbTLP1*) conferred transgenic tobacco plants resistance against *Verticillium* wilt [22], and two sea-island cotton (*G. barbadense*) dirigent-like genes (*Gbd1*, *Gbd2*) were induced by *V. dabliae* [15]. Overexpression of a *Verticillium* wilt resistance gene (*GbV*e) from cotton (*G. barbadense*) encoding a protein precursor in Arabidopsis enhanced plant resistance to *V. dahliae* [23]. On the other hand, silencing non-race-specific disease resistance 1 (*GhNDR1*) and mitogen-activated protein kinase kinases 2 (*GhMKK2*) genes compromised cotton resistance to the infection by *V. dahliae*, indicating that both genes are required for *Verticillium* resistance in cotton [40]. Similarly, our data demonstrate that overexpression of *GhDIR1* gene in cotton can delay the spread of fungal pathogen *V. dahliae* in transgenic plants.

Lignin is crucial in the development of vascular plants and is deposited mainly in vascular tissues. It provides additional strength and imperviousness to the cell wall during plant development. Furthermore, deposition of lignin in plants is considered to function as a physical barrier against invasion of pathogens [41]. There are many important enzymes involved in lignin biosynthesis pathway. Repression of caffeoyl coenzyme A *O*-methyltransferase (CCoAOMT) activity in transgenic poplar plants caused a significant decrease in total lignin content [42]. A recent study revealed that lignin content and composition are altered in transgenic plants of Norway spruce expressing a gene encoding cinnamoyl CoA reductase (CCR) in antisense orientation [43]. Suppression of *p*-coumaroyl-CoA 3′-hydroxylase in hybrid poplar impacted on lignin deposition [44]. Severe suppression of 4-coumarate-coenzyme A ligase (4CL) in the coniferous gymnosperm *Pinus radiate* plants resulted in a reduction in lignin content and changes in lignin composition [45]. In contrast, enhancing OsCCR1 activity induced a high accumulation of lignin in rice cell cultures [46]. A recent study indicated that lignin metabolism has a central role in the resistance of cotton to *V. dahlia*, the expression of many genes involved in lignin biosynthetic is regulated by *V. dahlia*, and the G/S ratio of cotton lignin may also affect plant resistance [47]. Likewise, GhDIR1 protein may be important for lignin biosynthesis pathway in cotton. Overexpression of the *GhDIR1* gene in cotton resulted in the increased lignin deposition in cell walls, and consequently, the transgenic

plants enhanced their defense against the invasion of fungal pathogen *V. dahliae*. Thus, this study may provide a new candidate gene for enhancing cotton tolerance to *Verticillium* wilt via genetic engineering.

Supplementary Data

Supplementary data are available at *ABBS* online.

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