The Cotton Lignin Biosynthetic Gene *Gh4CL30* Regulates Lignification and Phenolic Content and Contributes to Verticillium Wilt Resistance

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Verticillium wilt is a vascular disease causing tremendous damage to cotton production worldwide. However, our knowledge of the mechanisms of cotton resistance or susceptibility to this disease is very limited. In this study, we compared the defense transcriptomes of cotton (*Gossypium hirsutum*) cultivars Shidalukang 1 (*Verticillium dahliae* resistant, HR) and Junmian 1 (*V. dahliae* susceptible, HS) before and after *V. dahliae* infection, identified hub genes of the network associated with responses to *V. dahliae* infection, and functionally characterized one of the hub genes involved in biosynthesis of lignin and phenolics. We identified 6,831 differentially expressed genes (DEGs) between the basal transcriptomes of HR and HS; 3,685 and 3,239 of these DEGs were induced in HR and HS, respectively, at different time points after *V. dahliae* infection. KEGG pathway analysis indicated that DEGs were enriched for genes involved in lignin biosynthesis. In all, 23 hub genes were identified based on a weighted gene coexpression network analysis of the 6,831 DEGs and their expression profiles at different time points after *V. dahliae* infection. Knockdown of *Gh4CL30*, one of the hub genes related to the lignin biosynthesis pathway, by virus-induced gene silencing, led to a decreased content of flavonoids, lignin, and S monomer but an increased content of G monomer, G/S lignin monomer, caffeic acid, and ferulic acid, and enhanced cotton resistance to *V. dahliae*. These results suggest that *Gh4CL30* is a key gene modulating the outputs of different branches of the lignin biosynthesis pathway, and provide new insights into cotton resistance to *V. dahliae*.

Keywords: caffeic acid, ferulic acid, *Gh4CL30*, *Gossypium hirsutum*, lignin, transcriptome, Verticillium wilt, weighted gene co-expression network analysis

Cotton (*Gossypium hirsutum* L.), one of the most important fiber crops in the world, has crucial economic and social relevance, especially in Xinjiang Province, which is now the main area of cotton cultivation in China (Tian et al. 2017). Verticillium wilt is caused by *Verticillium dahliae*, which is a vascular fungal pathogen that can infect over 200 plant species, including members of the Malvaceae family such as cotton. Verticillium wilt in cotton causes serious economic losses (Cai et al. 2009; Deketelaere et al. 2017; Fradin and Thomma 2006). *V. dahliae* enters roots through root tips or small natural wounds. It then rapidly spreads through the xylem vessels to the stem and leaves of the infected plant. After successful infection, *V. dahliae* secretes exopolysaccharide that likely affects the plant's ability to transport water and nutrients, resulting in blockage of xylem vessels, leaf wilting, and death (Klosterman et al. 2009).

Plants use both physical and biochemical defenses against pathogen infection (Glazebrook 2005). Phenylpropanoid metabolites such as lignins and phenolic compounds play an essential role in plant disease resistance (Boerjan et al. 2003; Vogt 2010). In plant cells, lignin is produced by the phenylalanine/tyrosine metabolic pathway, one of the important branches of the phenylpropanoid pathway and part of a very complex metabolite network (Boerjan et al. 2003; Liu et al. 2018). Lignin formed by polymerization of monolignols provides a physical barrier to prevent entry and colonization of pathogen by reinforcing cell walls (Boerjan et al. 2003; Wang and Balint-Kurti 2016). Multiple enzymes are involved in lignin biosynthesis, including phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), hydroxycinnamoyl transferase (HCT), *p*-coumarate 3 hydroxylase (C3H), caffeic acid O-methyltransferase (COMT), Ferulate 5-hydroxylase (F5H), caffeoyl shikimate esterase (CSE), caffeoyl CoA O-methyltransferase (CCoAMT), cinnamoyl-CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD) (Boerjan et al. 2003; Vanholme et al. 2010).

Suppressing the expression of lignin biosynthesis-related genes such as *COMT1* and *CAD* in *Arabidopsis* and *PAL4* in rice enhanced plant susceptibility to pathogens (Tonnessen et al. 2015; Tronchet et al. 2010). Wheat plants with silenced *TmPAL*, *TmCOMT*, *TmCCoAMT*, or *TmCAD* decreased resistance to *Blumeria graminis* f. sp. *tritici*. Cosilencing two of these

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genes (*TmPAL+TmCAOMT*, *TmPAL+TmCAD*, or *TmCAOMT+TmCAD*) further enhanced disease susceptibility compared with silencing individual genes (Bhuiyan et al. 2009). Lignin biosynthesis-related genes have also been linked to defense against diseases in alfalfa, tobacco, potato, and cotton (Gill et al. 2018; Hu et al. 2019; Shadle et al. 2003; Xu et al. 2011). 4CL is the third key enzyme in the phenylpropanoid metabolic pathway which contributes to biosynthesis of lignin and flavonoids (Vogt 2010). Previous studies have shown that the expression level of 4CL is strongly induced by pathogens in different plants (Oliveira et al. 2015; Sun et al. 2013; Xu et al. 2011). However, except for a 4CL-like gene, *OsAAE3* (Liu et al. 2017), the function of 4CL in plant disease resistance is rarely reported.

Inhibition of steps of the phenylpropanoid pathway may lead to accumulation or reduction of phenolic compounds (Konig et al. 2014). Phenolic compounds generated in the various steps of the phenylpropanoid pathway have a wide range of roles in plants, including growth, development, reproduction, adaptation, and protection from biotic and abiotic stresses (Lavhale et al. 2018; Mierziak et al. 2014). The function of phenolic compounds in plant-pathogen interactions is well known, by which they have strong antibacterial and antifungal qualities (Lanoue et al. 2010). Some reports have provided evidence to suggest that phenolic compounds play important defense roles in cotton's response to *V. dahliae* infection, whereas only a few specific monophenols have been functionally characterized (Gao et al. 2013; Y. Zhang et al. 2017).

G. hirsutum and *G. barbadense* are the two major allotetraploid cotton species, contributing more than 90% of worldwide fiber production (Wang et al. 2019). Although both *G. hirsutum* and *G. barbadense* originated from one hybridization event between the ancestor of *G. herbaceum* (A1) or *G. arboreum* (A2) and the ancestor of *G. raimondii* (D5) approximately 1 million to 1.5 million years ago, they have great differences in fiber quality, disease resistance, production, and other traits (Wendel 1989). *G. barbadense* varieties often show high resistance to *V. dahliae* but few *G. hirsutum* varieties do (Zhang et al. 2013). A number of RNA sequencing (RNA-seq) experiments have been carried out in cotton to investigate the defense transcriptome against *V. dahliae* and the mechanism of cotton-*V. dahliae* interaction (Zhang et al. 2013). Most investigations had a focus on resistant *G. barbadense* accessions (Xu et al. 2011, 2014; Zhang et al. 2013) or compared the differences between resistant *G. barbadense* accessions and susceptible *G. hirsutum* accessions (Gao et al. 2013; Sun et al. 2013), although one study compared the defense transcriptomes of *V. dahliae*-resistant and *V. dahliae*-susceptible *G. hirsutum* varieties (W. Zhang et al. 2017). The mechanisms of the molecular interactions that confer high levels of resistance versus susceptibility in the cotton-*V. dahliae* interaction remain to be fully elucidated.

In this study, we compared the basal and defense transcriptomes of a *V. dahliae*-resistant (HR) and a *V. dahliae*-susceptible (HS) cotton (*G. hirsutum*) variety, and identified enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and hub genes associated with cotton response to *V. dahliae* infection. Functional characterization demonstrated that silencing *Gh4CL30*, one of the hub genes involved in the lignin biosynthesis pathway, was able to enhance *V. dahliae* resistance, which could be achieved by increasing the ratio of the G/S lignin monomer and the contents of caffeic acid and ferulic acid.

RESULTS

Characterization of the two cotton cultivars in response to *V. dahliae* infection.

We initially conducted disease assays to confirm the level of *V. dahliae* response of the two *G. hirsutum* cultivars (Shidalukang

1: HR and Junmian 1: HS) to be used. At 21 days postinfection (dpi), we observed significant difference of disease response between HR and HS. All HS seedlings exhibited strong disease symptoms and ultimately died, whereas the majority of HR seedlings showed no obvious disease symptoms (Fig. 1A). Both the ratio of diseased plants and disease index of HS were significantly higher than those of HR at 14 and 21 dpi (Fig. 1B and C). Moreover, fungal recovery assays, vascular browning observation, and fungal biomass analysis all indicated that the level of *V. dahliae* resistance of HR was much higher than that of HS (Fig. 1D to F).

HR and HS showed different basal transcriptome.

We performed RNA-seq using samples from 0, 12, 24, and 48 h postinoculation (hpi) from HR and HS. In total, 1.20 billion clean reads (approximately 180.35 Gb of data) were obtained (Supplementary Table S2). Across all samples, the percentage of nucleotides with a quality score above 20 was over 97.67%, and the GC percentage ranged from 43.03 to 43.69% (Supplementary Table S2). Approximately 95% of the clean reads were mapped to the *G. hirsutum* reference genome (TM-1) (Zhang et al. 2015). Of the approximately 5% clean reads that did not align to the TM-1 genome, only 2.53 to 4.63% could be aligned to the *V. dahliae* genome (Klosterman et al. 2011) (Supplementary Table S3).

To investigate whether the basal transcriptomes of HR and HS are related to their different disease response, we compared the 0-hpi transcriptomes (i.e., uninfected by *V. dahliae*) of HR and HS and identified 6,831 differentially expressed genes (DEGs) between HR and HS using DESeq (adjusted *P* value < 0.05). Of these DEGs, 2,866 (type I) and 3,965 (type II) had significantly lower and higher expression levels, respectively, in HS than in HR (Fig. 2A; Supplementary Tables S4 and S5). We selected six type I DEGs (HR > HS) for confirmation using reverse-transcription quantitative PCR (RT-qPCR). As expected, the expression levels of all six genes (*GhABC1*, *GhABC2*, *GhMLP1*, *GhMLP2*, *GhWAK1*, and *GhRPS1*) in the roots or stems were higher in HR than in HS (Supplementary Fig. S1A and B), suggesting the reliability of the RNA-seq results.

To predict the functions of the two types of DEGs, KEGG pathway analysis was performed for each type of DEG. The result showed that the type I DEGs enriched for only six KEGG pathways (*P* value < 0.05) (Fig. 2B; Supplementary Table S6), including DNA replication, carbon fixation in photosynthetic, pyrimidine metabolism, pentose phosphate pathway, mismatch repair, and flavonoid biosynthesis. For type II DEGs, 11 KEGG pathways were found to be enriched (Fig. 2B; Supplementary Table S7). Consistent with other reports, some of these enriched pathways were associated with plant responses to *V. dahliae* infection; for example, flavonoid biosynthesis and phenylpropanoid biosynthesis (Guo et al. 2017; Xu et al. 2011). Genes related to the flavonoid biosynthesis pathway were enriched in both HR and HS; however, the phenylpropanoid biosynthesis pathway was enriched only in HS (Fig. 2B).

Genes response to stimulus and stresses were highly expressed in HR.

To further explore the functions of DEGs, the two types of DEGs were annotated based on functional gene ontology (GO) using the default Blast2GO parameters. Of the top 20 GO terms (*P* value < 0.05), none overlapped between the type I and type II DEGs (Fig. 2C; Supplementary Tables S8 and S9). Overall, processes involved in DNA binding (GO:0003677) and response to stimulus (GO:0050896) were the most enriched in type I DEGs (Fig. 2C). Interestingly, in total, 292 genes responsive to stimulus (GO:0050896; *P* = 0.013) and 148 genes

associated with stress responses (GO:0006950; $P = 0.005$) were discovered in HR but not in HS (Fig. 2C), including five ABC transporters, two major latex proteins (MLPs), one wall-associated receptor-like kinase, and several disease resistance proteins (nucleotide binding leucine-rich repeat protein and nucleotide binding adaptor shared by APAF-1, resistance proteins, and CED-4 domain protein). In type II DEGs, the two major GO terms were metabolic process (GO:0008152) and catalytic activity (GO:0003824) (Fig. 2C).

Identification of DEGs in response to *V. dahliae* infection.

To examine the changes of the basal transcriptome of HR and HS in response to *V. dahliae* infection, we performed pairwise transcriptome comparisons between the infected (12, 24, and 48 hpi) and uninfected (0 hpi) HR and HS. In HR, 5,146, 8,011, and 9,014 DEGs were identified at 12, 24, and 48 hpi, respectively (Supplementary Fig. S2A). The numbers of DEGs identified at 12, 24, and 48 hpi in HS were 6,970, 7,754, and 6,392, respectively (Supplementary Fig. S2B). In total, 13,250

and 12,602 nonredundant DEGs were identified in HR and HS, respectively, and 5,930 were common in HR and HS (Supplementary Fig. S2C).

Of the type I and II basal DEGs identified between HR and HS, 782 and 943, respectively, were not significantly changed in response to *V. dahliae* infection. Of the type I DEGs responsive to *V. dahliae* infection, 903 and 461 responded in only HR and only HS, respectively, and 720 responded in both HR and HS (Fig. 2D). Likewise, following *V. dahliae* infection, 946 and 960 type II DEGs responded in only HR and only HS, respectively, and 1,098 responded in both HR and HS (Fig. 2D). Of the total 6,831 type I and II DEGs, 3,685 and 3,239 responded to *V. dahliae* infection in HR and HS, respectively (Fig. 2D; Supplementary Tables S10 and S11). These genes were mapped to 108 and 114 KEGG pathways, with 13 and 12 enriched pathways (P value < 0.05), respectively. Eight of them were enriched in both HR and HS (Fig. 2E; Supplementary Tables S12 and S13). Notably, the phenylpropanoid biosynthesis pathway was only enriched in HR.

Fig. 1. Levels of resistance of resistant Shidalu kang 1 (HR) and susceptible Junmian 1 (HS) to *Verticillium dahliae*. **A**, Phenotypes of HR and HS at 21 days postinfection (dpi) with *V. dahliae*. **B** and **C**, Ratio of diseased plants and disease index of HR and HS at 14 and 21 dpi. **D**, Comparison of disease symptoms in stems of HR and HS at 14 dpi. **E**, Fungal isolation in the stem sections from HR and HS at 14 dpi. Stems were plated on potato dextrose agar medium. Photos were taken after 7 days of culture at 25°C. **F**, Quantitative PCR analysis of the relative fungal biomass in stems of HR and HS at 14 dpi. Data are the mean $\pm$ standard error of three independent biological replicates, and asterisks indicate significant differences between groups determined using the Student's t test (* and ** indicate $P < 0.05$ and 0.01 , respectively).

Lignin biosynthesis pathways were activated in both HR and HS cultivars after *V. dahliae* infection.

The phenylpropanoid metabolic pathway plays an important role in cotton defense against *V. dahliae* (Xu et al. 2011). Eleven classes of lignin biosynthesis-related genes were identified in the phenylpropanoid biosynthesis pathway. All of these genes were significantly induced in HR or HS upon *V. dahliae* infection, including six *GhPAL*, one *GhC4H*, four *Gh4CL*, two *GhHCT*, two *GhC3H*, six *GhCOMT*, one *GhF5H*, two *GhCSE*, seven *GhCCoAOMT*, two *GhCCR*, and two *GhCAD* (Fig. 3A; Supplementary Table S14). Based on their expression profiles at 0 and 48 hpi in HR and HS, these genes could be clustered into two groups. The expression levels of the first group of genes were consistently higher in HS than in HR at both time points (Supplementary Fig. S3), while the expression levels of the second group of genes were higher in HS than in HR at 0 hpi but were variable between HR and HS at 48 hpi (with a similar expression level in most cases) (Supplementary Fig. S4).

When stained with phloroglucinol, the stem cross-sections of HS plants exhibited deeper red than HR at 0 and 48 hpi (Fig. 3B). Consistent with this observation, HS plants had a significantly higher level of lignin than HR plants at both 0 and 48 hpi (Fig. 3C). In both HS and HR plants, the lignin content was significantly higher at 48 hpi than at 0 hpi (Fig. 3C). Of the two major lignin monomers, the contents of the G and S monomers were significantly higher in HS than in HR at both 0 and 48 hpi, and also significantly increased in both HS and HR plants following *V. dahliae* infection (Fig. 3D, E). Compared with 0 hpi, the G/S ratio was significantly increased and decreased in HR and HS plants, respectively, at 48 hpi (Fig. 3F).

Weighted gene coexpression network analysis.

To determine the potential role and the response of the basal transcriptome upon *V. dahliae* infection, we did weighted gene coexpression network analysis (WGCNA) analysis using the 6,831 DEGs identified between HR and HS at 0 hpi. These DEGs were grouped into 11 different modules based on pairwise correlation analysis of gene expression across all samples (Fig. 4). Five modules (Fig. 4B, indicated by a red underline) had a *P* value < 0.01 and a corresponding correlation coefficient > 0.5 in at least one of the samples.

The purple module included 115 genes that were induced faster in HS than in HR (Supplementary Table S15). The expression levels of the 1,195 genes included in the brown module were quickly decreased in HS after *V. dahliae* infection, whereas the expression levels of the blue module genes were quickly reduced in HR after *V. dahliae* infection (Supplementary Table S15). The yellow module included 615 genes that were slightly decreased at 24 and 48 hpi in HS but increased in HR at 24 hpi (Supplementary Table S15). The expression levels of the green module genes were rapidly decreased at 12 hpi in HS but increased in HS at 24 and 48 hpi (Supplementary Table S15). In total, 21 hub genes were identified based on the criteria of eigengene-based connectivities > 0.9 and protein-protein interaction > 5 (Table 1).

Expression pattern of *Gh4CL30*.

According to KEGG, WGCNA, and lignin content analyses, several genes of the lignin biosynthesis pathway are promising candidate genes related to the different *V. dahliae* resistance levels between HR and HS, including *Gh_D10G0473*, one of the hub genes identified based on gene network analysis. We have systematically analyzed the cotton *4CL* gene family and named *Gh_D10G0473* as *Gh4CL30* (Sun et al. 2020).

Fig. 2. Basal transcriptomes of resistant Shidalukang 1 (HR) and susceptible Junmian 1 (HS) (from *Verticillium dahliae*-uninfected plants) and their responses to *V. dahliae* infection. **A**, Heatmap of the 6,831 differentially expressed genes (DEGs) identified in the basal transcriptomes of HR and HS. **B** and **C**, Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology analyses of the 2,866 and 3,965 basal DEGs with a higher expression level in HR and HS, respectively. **D**, Venn diagram showing the number of the basal DEGs that were induced after *V. dahliae* infection in HR and HS. **E**, KEGG analysis of the basal DEGs that were significantly induced in HR and HS.

Fig. 3. Expression profiles of the lignin biosynthesis-related genes, comparison of lignin content, and lignin monomer in resistant Shidialukang 1 (HR) and susceptible Junmian 1 (HS) before and after *Verticillium dahliae* infection. **A**, Transcript profiling of the 34 differentially expressed genes (DEGs) involved in the lignin biosynthesis pathways in HR and HS. PAL = phenylalanine ammonia-lyase, C4H = cinnamate 4-hydroxylase, 4CL = 4-coumarate: CoA ligase, C3H = p-coumarate 3 hydroxylase, HCT = hydroxycinnamoyl transferase, CSE = caffeoyl shikimate esterase, F5H = ferulate 5-hydroxylase, CCR = cinnamoyl CoA reductase, CAD = cinnamyl alcohol dehydrogenase, COMT = caffeic acid 3-O-methyltransferase, and CCoAOMT = caffeoyl-CoA O-methyltransferase. **B**, Histochemical analysis (stained with phloroglucinol-HCl) of lignin in the stem cross-sections of HR and HS plants at 0 and 48 h postinoculation (hpi). Scale bars = 300 μ m. The content of **C**, total lignin; **D**, G monomer; and **E**, S monomer in the stems of HR and HS at 0 and 48 hpi. **F**, The G/S ratio in the stems of HR and HS at 0 and 48 hpi. Data are the mean $\pm$ standard error of three independent biological replicates, and different letters indicate significant differences ($P < 0.05$) between groups determined using the Duncan's multiple range test.

To investigate the role of *Gh4CL30* in *V. dahliae* resistance, we first determined the expression pattern of *Gh4CL30* in different tissues of HR and HS. *Gh4CL30* had a higher expression level in *V. dahliae*-susceptible varieties than in *V. dahliae*-resistant varieties in all three tissues analyzed. Among the three tissues, the expression level of *Gh4CL30* was higher in stems than in roots and leaves (Fig. 5A). Time course expression analysis in both roots and stems revealed that, in response to *V. dahliae* infection, the expression level of *Gh4CL30* was significantly upregulated only in roots at 24 hpi and in both roots and stems at 48 and 72 hpi in the two cultivars (Fig. 5B and C). These results suggested that *Gh4CL30* might be a negative regulator of *V. dahliae* resistance.

Virus-induced gene silencing mediated silencing of *Gh4CL30* enhanced cotton resistance to *V. dahliae*.

If *Gh4CL30* acts as a negative regulator of *V. dahliae* resistance, we expected to see enhanced *V. dahliae* resistance in HS when *Gh4CL30* is silenced. We used virus-induced gene silencing (VIGS) to silence *Gh4CL30* and successfully generated VIGS plants, as demonstrated by the yellowing phenotype observed on the newly developing true leaves in the control *TRV:GhCHLI* plants (Supplementary Fig. S5). RT-qPCR analysis of *Gh4CL30* in *TRV:00* and *TRV:Gh4CL30* plants 2 weeks after infiltration confirmed significant reduction of the transcript level of *Gh4CL30* in *TRV:Gh4CL30* plants (Fig. 6A). Pathogen infection experiments indicated that the *TRV:Gh4CL30* plants had a significantly increased level of *V. dahliae* resistance (Fig. 6B), as demonstrated by less severe disease symptoms and diseased plants, and lower disease index was observed in the *TRV:Gh4CL30* plants (Fig. 6C and D), as well as fewer fungal colonies recovered from stems of the *TRV:Gh4CL30* plants and significantly lower accumulation of fungal biomass in the *TRV:Gh4CL30* plants (Fig. 6E and F).

It is noteworthy that stems of the mock *TRV:00* plants did not show red-brown color in the xylem, whereas the *TRV:Gh4CL30* stems did. Upon *V. dahliae* infection, both *TRV:00* and *TRV:*

Gh4CL30 plants exhibited a red-brown phenotype in the xylem of stems, with a stronger color observed in the *TRV:Gh4CL30* plants (Supplementary Fig. S6), and the intensity of the red-brown

Table 1. Candidate hub genes associated with response to *Verticillium dahliae* inoculation in Shidalukang 1 and Junmian 1

Gene name	Description	KME value ^a
Purple module		
<i>Gh_D10G0473</i>	4-Coumarate-CoA ligase 1	0.92
<i>Gh_A13G2072</i>	Cytochrome P450 98A2	0.90
Brown module		
<i>Gh_A01G1981</i>	Ammonium transporter 2	0.94
<i>Gh_A08G1891</i>	Glutamate dehydrogenase 3	0.90
Yellow module		
<i>Gh_A12G1327</i>	ATP-citrate synthase α chain protein 1	0.93
Blue module		
<i>Gh_D11G1150</i>	DNA replication licensing factor MCM7	0.95
<i>Gh_A11G0031</i>	Thaumatococcus-like protein	0.95
<i>Gh_A11G3071</i>	DNA replication licensing factor MCM7	0.93
<i>Gh_D08G0168</i>	Origin of replication complex subunit 1B	0.93
<i>Gh_A13G0286</i>	DNA replication licensing factor MCM4	0.93
<i>Gh_D03G0910</i>	Replication protein A 70-kDa DNA-binding subunit B	0.92
<i>Gh_D05G3787</i>	PsbP-like protein 1, chloroplastic	0.92
<i>Gh_A02G1196</i>	Pleiotropic drug resistance protein 1	0.92
<i>Gh_A03G0625</i>	Replication protein A 70-kDa DNA-binding subunit B	0.91
<i>Gh_A07G1821</i>	Cell division control protein 45 homolog	0.91
Green module		
<i>Gh_D06G0003</i>	Hexokinase-2	0.97
<i>Gh_D01G0800</i>	Phosphoglycerate kinase	0.95
<i>Gh_A05G1529</i>	Glyceraldehyde-3-phosphate dehydrogenase B	0.95
<i>Gh_A10G0618</i>	Glyceraldehyde-3-phosphate dehydrogenase A	0.94
<i>Gh_A05G1564</i>	Plastocyanin A	0.94
<i>Gh_A06G0047</i>	Hexokinase-2	0.93

^a KME = eigengene-based connectivities.

Fig. 4. Weighted gene coexpression network analysis (WGCNA) based on the differentially expressed genes (DEGs) identified between resistant Shidalukang 1 (HR) and susceptible Junmian 1 (HS) at 0 h postinoculation (hpi) and their expression profiles at 12, 24, and 48 hpi. **A**, Hierarchical cluster tree showing coexpression modules identified by WGCNA. Each leaf in the tree represents one gene. Each major tree branch corresponds to a distinct module. In total, 6,829 DEGs were grouped into 10 coexpression modules labeled with different colors. The DEGs that did not show a coexpression relationship were assigned into the gray module. **B**, Relationships between the coexpression modules and samples. Each row corresponds to a module, labeled with the same color as in **A**. The corresponding correlation and *P* value were annotated in each cell. Modules that had a high degree of correlation in at least one time point in either of the two varieties are indicated by a red underline.

color decreased upward in the xylem of stems (Supplementary Fig. S7).

Silencing of *Gh4CL30* had variable effect on the accumulation of different lignin monomers.

It is known that *4CLs* are key genes involved in the biosynthesis of lignins (Vanholme et al. 2010). We compared the lignin content in the stems of the *TRV:00* and *TRV:Gh4CL30* plants. Phloroglucinol staining of stems suggested that the *TRV:Gh4CL30* plants accumulated less lignin than the *TRV:00* plants regardless of whether or not the plants were inoculated with *V. dahliae* (Fig. 7A). Consistently, significantly lower amount of total lignin was detected in the *TRV:Gh4CL30* plants than in

the *TRV:00* plants in both mock and *V. dahliae* inoculation treatments (Fig. 7B). In agreement with these observations, the expression levels of four lignin biosynthesis-related genes, including *GhCOMT1*, *GhCCR1*, *GhCCR2*, and *GhCAD1*, were significantly downregulated in the *TRV:Gh4CL30* plants compared with the *TRV:00* plants in both mock and *V. dahliae* inoculation treatments. The expression levels of two other lignin biosynthesis-related genes, *GhC4H1* and *GhCCoAOMT1*, were reduced only in the stems from *V. dahliae*-infected plants but not in the stems from uninfected plants (Fig. 7C to H). Given the importance of different lignin monomers in plant defense against *V. dahliae* infection (Xu et al. 2011), we further measured the content of the G, S, and H lignin monomers. In both

Fig. 5. Expression pattern of *Gh4CL30* in different cotton tissues and in response to *Verticillium dahliae* inoculation. **A**, Expression profiling of *Gh4CL30* in roots, stems, and leaves in resistant Shidialukang 1 (HR) and susceptible Junmian 1 (HS). Roots, stems, and leaves were sampled from two-leaf-stage seedlings grown in a greenhouse. **B**, Expression patterns of *Gh4CL30* in the roots of HS and HR at 0, 12, 24, 48, and 72 h postinoculation (hpi). GhUBQ7 was used as the control. **C**, Expression patterns of *Gh4CL30* in the stems of HS and HR at 0, 12, 24, 48, and 72 hpi. GhUBQ7 was used as the control. Data are the mean $\pm$ standard error of three independent biological replicates, and asterisks indicate significant differences between groups determined using the Student's *t* test (* and ** indicate $P < 0.05$ and 0.01 , respectively).

Fig. 6. Silencing of *Gh4CL30* in Junmian 1 (HS) improved resistance to *Verticillium dahliae*. **A**, Reverse-transcription quantitative PCR (RT-qPCR) analysis of *Gh4CL30* expression in the *TRV:00* and *TRV:Gh4CL30* plants. Total RNA was isolated from stems at 10 days after infiltration with virus-induced gene silencing vector. GhUBQ7 was used as the control. **B**, Phenotypes of the *TRV:00* and *TRV:Gh4CL30* plants at 14 days postinfection (dpi). **C** and **D**, Ratio of diseased plants and disease index for the *TRV:00* and *TRV:Gh4CL30* plants at 14 and 21 dpi. **E**, Fungal isolation in the stem sections from *TRV:00* and *TRV:Gh4CL30* plants at 14 dpi. Stems were plated on potato dextrose agar medium. Photos were taken after 7 days of culture at 25°C. **F**, qPCR analysis of the relative fungal biomass in stems of the *TRV:00* and *TRV:Gh4CL30* plants at 14 and 21 dpi. Data are the mean $\pm$ standard error of three independent biological replicates, and asterisks indicate significant differences between groups determined using the Student's *t* test (* and ** indicate $P < 0.05$ and 0.01 , respectively).

mock and *V. dahliae* infection treatments, the content and the proportion of the G unit were higher in the *TRV:Gh4CL30* plants than in the *TRV:00* plants; by contrast, the content and the proportion of the S unit were lower in the *TRV:Gh4CL30*

plants than in the *TRV:00* plants. The content and the proportion of the less abundant H unit remained stable. As a result, the G/S ratio was higher in the *TRV:Gh4CL30* plants than in the *TRV:00* plants, particularly in the *V. dahliae* treatment (Table 2). The

Fig. 7. Comparison of lignin content and expression of lignin biosynthesis-related genes in *TRV:00* and *TRV:Gh4CL30* plants at 48 h postinoculation (hpi) (mock versus *Verticillium dahliae* infection). **A**, Histochemical analysis of lignin in the stem cross-sections from *TRV:00* and *TRV:Gh4CL30* plants. The hand-cut stem was stained with phloroglucinol-HCl to detect lignin. Scale bars = 300 μ m. **B**, Comparison of the total lignin content in the stems of *TRV:00* and *TRV:Gh4CL30* plants after 48-h treatment of *V. dahliae* or sterile distilled water. **C** to **H**, Reverse-transcription quantitative PCR (RT-qPCR) analysis of lignin biosynthesis-related genes in *TRV:00* and *TRV:Gh4CL30* plants after 48-h treatment of *V. dahliae* or sterile distilled water. Data are the mean $\pm$ standard error of three independent biological replicates, and asterisks indicate significant differences between groups determined using the Student's *t* test (* and ** indicate $P < 0.05$ and 0.01 , respectively).

decreased total lignin content observed in *TRV:Gh4CL30* was mainly due to reduction of the S unit (Fig. 7B; Table 2). Nevertheless, compared with the mock treatment, *V. dahliae* induced accumulation of lignin in both *TRV:00* and *TRV:Gh4CL30* plants (Fig. 7B; Table 2). These results suggest that knockdown of the expression level of *Gh4CL30* had different effects on the production of the G and S lignin monomers but *V. dahliae* infection consistently enhanced the production of all lignin monomers.

Silencing of *Gh4CL30* repressed the salicylic acid and jasmonic acid signaling pathways and flavonoid biosynthesis.

Salicylic acid (SA) and jasmonic acid (JA) are two of the major phytohormones that play a vital role in plant defense against pathogen infection (Shaban et al. 2018). To investigate the effect of silencing *Gh4CL30* on the SA and JA biosynthesis or signaling pathways, we compared the SA and JA concentrations in the stems of the *TRV:00* and *TRV:Gh4CL30* plants. The content of SA was significantly lower in *TRV:Gh4CL30* than in *TRV:00* in both uninfected and infected treatments (Supplementary Fig. S8A); however, the reduction of JA content in *TRV:Gh4CL30* was observed only in the mock treatment (Supplementary Fig. S8B). Compared with the *TRV:00* plants, the *TRV:Gh4CL30* plants had a variable change in the expression level of the SA signaling or biosynthesis genes in the mock treatment but showed a consistently significant reduction of all four genes upon *V. dahliae* infection (48 hpi) (Supplementary Fig. S8C to F). The two JA biosynthesis or signaling genes (*GhAOS1* and *GhPR4*) were significantly inhibited in the *TRV:Gh4CL30* plants compared with the *TRV:00* plants in both the mock and *V. dahliae* infection treatments (Supplementary Fig. S8G and H). These results suggest that downregulation of *Gh4CL30* tends to repress accumulation of SA and JA that is further affected by *V. dahliae* infection.

4CLs are also involved in the biosynthesis of flavonoids (Lavhale et al. 2018). Previous studies have reported that increased contents of flavonoids inhibited colony growth of *V. dahliae* (Hu et al. 2018). We found that the diameters of *V. dahliae* colonies growing on the medium treated with the methanolic extracts from the *TRV:Gh4CL30* plants were significantly smaller than those treated with the extracts from the *TRV:00* plants (Fig. 8A and B). However, we observed a significant decrease of flavonoids in the *TRV:Gh4CL30* plants compared with the *TRV:00* plants in both the mock and *V. dahliae* inoculation treatments (Supplementary Fig. S9A). The expression levels of four flavonoid biosynthesis-related genes, including *GhANR1*, *GhCHI1*, *GhLAR1*, and *GhANS1*, were significantly reduced in the *V. dahliae*-infected *TRV:*

Gh4CL30 plants, although no significant difference was evident between the uninfected *TRV:Gh4CL30* and *TRV:00* plants (Supplementary Fig. S9B to E). These results suggest that the substances from *TRV:Gh4CL30* that inhibited the growth of the *V. dahliae* colony are most likely not flavonoids.

Altered phenolic metabolism in the *Gh4CL30*-silenced plants was associated with resistance to *V. dahliae*.

Reports have suggested that the phenolic precursors of lignin biosynthesis, including ferulic acid and caffeic acid, have anti-fungal effects (Lanoue et al. 2010; Lavhale et al. 2018). Thus, we quantified the content of four phenolics in the lignin biosynthesis pathway, including cinnamic acid, coumalic acid, caffeic acid, and ferulic acid, in the stems of the *TRV:00* and *TRV:Gh4CL30* plants. Under the mock treatment, the contents of ferulic acid and caffeic acid were 9.0- and 6.1-fold higher, respectively, in *TRV:Gh4CL30* than in *TRV:00* (Fig. 8C and D). At 48 hpi, *TRV:Gh4CL30* showed an even higher increase of the two phenolics, at 10.4- and 32.3-fold for ferulic acid and caffeic acid, respectively (Fig. 8C and D). No significant change was observed for coumalic acid and cinnamic acid (Fig. 8E and F). These results suggest that accumulation of ferulic acid and caffeic acid might be associated with the enhanced *V. dahliae* tolerance observed in *TRV:Gh4CL30*.

We investigated the effect of ferulic acid and caffeic acid on the growth of *V. dahliae* (strain V991) using four different concentrations (10 $\mu\text{g ml}^{-1}$, 100 $\mu\text{g ml}^{-1}$, 1 mg ml^{-1} , and 10 mg ml^{-1}) of these two phenolics. Three of the four concentrations (except 10 $\mu\text{g ml}^{-1}$) were found to significantly inhibit the growth of *V. dahliae* colony, and the higher the concentration, the stronger the inhibition (Fig. 8G and H; Supplementary Fig. S10). Furthermore, a mixture of ferulic acid and caffeic acid seemed to have a stronger inhibition compared with either single phenolic (Fig. 8G and H; Supplementary Fig. S10). These findings indicated that the higher levels of ferulic acid and caffeic acid contribute to the increased resistance level of the *TRV:Gh4CL30* plants against *V. dahliae* infection.

DISCUSSION

In this study, we used comparative transcriptomics to investigate the molecular mechanism underlying the basal and pathogen-induced responses in the interaction between cotton and *V. dahliae*. We identified *Gh4CL30* to be one of the hub genes that were differentially expressed between cultivars resistant or susceptible to *V. dahliae*. *Gh4CL30* regulates lignification and phenolic content and negatively contributes to *V. dahliae* resistance. We also found that a higher ratio of the G/S lignin monomer is more important than the total lignin content in cotton resistance to *V. dahliae*. The present study

Table 2. Comparison of individual lignin monomers in the *Gh4CL30*-silenced and *TRV:00* plants in Junmian 1^x

Sample	Monomer composition (mg g ⁻¹ dry weight)			Lignin ^y	Ratio of monomer composition			G/S ^z
	G	S	H		G	S	H	
Mock								
<i>TRV:00</i>	11.50 ± 0.25 c	10.39 ± 0.15 b	0.54 ± 0.05 b	22.43 ± 0.65 b	0.51	0.46	0.02	1.11
<i>TRV:Gh4CL30</i>	12.43 ± 0.28 b	8.49 ± 0.15 c	0.62 ± 0.02 a	21.53 ± 0.41 c	0.58	0.39	0.03	1.46
Inoculated								
<i>TRV:00</i>	12.20 ± 0.14 b	12.55 ± 0.23 a	0.52 ± 0.02 b	25.27 ± 0.71 a	0.48	0.50	0.02	0.97
<i>TRV:Gh4CL30</i>	13.71 ± 0.34 a	8.95 ± 0.21 c	0.53 ± 0.02 b	23.19 ± 0.44 b	0.59	0.39	0.02	1.53

^x The contents of lignin monomer composition in the stem of *TRV:00* and *TRV:Gh4CL30* plants after 48-h treatment of *Verticillium dahliae* or sterile distilled water. Data are the mean ± standard error of three independent biological replicates, and different letters indicate significant differences ($P < 0.05$) between groups determined using the Duncan's multiple range test.

^y Total lignin content.

^z G/S ratio.

improves our understanding of the functions of *Gh4CL30* and provides new insights about the resistance mechanism employed by cotton against *V. dahliae*.

Both basal and pathogen-induced transcriptomes play an important role in cotton defense against *V. dahliae*.

When doing transcriptome comparison, most researches usually focus on differentially expressed genes between infected and uninfected samples of resistant and susceptible cultivars (Gao et al. 2013; Sun et al. 2013; Xu et al. 2011, 2014; Zhang et al. 2013), and the basal transcriptomic difference between susceptible and resistant cultivars is largely overlooked. In a recent study in olive, the *V. dahliae* tolerance and susceptibility were linked to differences in the basal transcriptomes (Leyva-Pérez et al. 2018). Here, we found 6,831 DEGs in uninfected roots and stems between HR and HS (Fig. 2A; Supplementary Tables S4 and S5), of which 2,866 and 3,965 had expression levels higher in HR and HS, respectively (Fig. 2D; Supplementary Tables S4 and S5). These basal DEGs may contribute to the resistance and susceptibility of HR and

HS. Many genes highly expressed in uninfected HR were involved in response to stimulus and stress (Fig. 2C), including two ABC transporters, two major latex proteins, one wall-associated receptor-like kinase, and several disease resistance proteins. Consistently, recent studies showed that silencing *GhABCF5* or *GhMLP28* in cotton led to a significantly decreased resistance to *V. dahliae* (Dong et al. 2019; Yang et al. 2015). The plants with a mutation in the gene (*ZmWAK-RLK1*) encoding a wall-associated receptor-like kinase were more susceptible to northern corn leaf blight than wild-type plants in both the field and greenhouse conditions (Hurni et al. 2015).

Numerous studies have demonstrated that plants restrict pathogen spread in the vascular structure by changing the lignin monomer composition and lignification (Hu et al. 2018; Xu et al. 2011). We also found a large number of lignin biosynthesis-related genes that were significantly induced in both HR and HS after *V. dahliae* infection (Supplementary Figs. S3 and S4). Consequently, both HR and HS plants showed a higher level of total lignin, due to accumulation of the G and S monomers, after *V. dahliae* infection (Fig. 3B to E). Similar

Fig. 8. Silencing *Gh4CL30* enhances cotton resistance to *Verticillium dahliae* correlates with accumulation of caffeic acid and ferulic acid. **A** and **B**, The fungus was grown for 10 days on potato dextrose agar (PDA) plates with methanolic extracts (the final concentration was 2% [vol/vol]) from the stems of *TRV:00* and *TRV:Gh4CL30* plants and the diameter of the colony was determined. Scale bars = 2 cm. Data are the mean $\pm$ standard error (SE) of three independent biological replicates, and different letters indicate significant differences ($P < 0.05$) between groups determined using the Duncan's multiple range test. Comparison of **C**, ferulic acid; **D**, caffeic acid; **E**, cinnamic acid; and **F**, coumalic acid in the stem of *TRV:00* and *TRV:Gh4CL30* plants after 48-h treatment of *V. dahliae* or sterile distilled water. Data are the mean $\pm$ SE of three independent biological replicates, and asterisks indicate significant differences between groups determined using the Student's *t* test (* and ** indicate $P < 0.05$ and 0.01 , respectively). **G** and **H**, Effects of ferulic acid (FA) and caffeic acid (CA) on colony growth ($n = 5$). The photograph was taken after growing the fungus for 12 days on the PDA plates with FA or CA at $100 \mu\text{g ml}^{-1}$. Scale bars = 2 cm. Data are the mean $\pm$ SE of three independent biological replicates, and different letters indicate significant differences ($P < 0.05$) between groups determined using the Duncan's multiple range test.

results were reported in the *V. dahliae*-tolerant *G. barbadense* accession and *V. dahliae*-susceptible *G. hirsutum* accession (Xu et al. 2011). These findings suggest that activation of the lignin biosynthesis pathways is associated with pathogen-induced resistance in both *V. dahliae*-resistant and -susceptible cotton. Previous studies showed that resistant cotton plants accumulated more lignin than susceptible cotton plants after *V. dahliae* inoculation and that the lignin content was positively correlated with *V. dahliae* resistance (Xu et al. 2011; Zhang et al. 2019). However, we found that HR had a significantly lower level of lignin than HS in both mock and *V. dahliae* infection treatments, despite *V. dahliae*-induced accumulation of lignin (Fig. 3B to E). Similarly, decreased lignin content but increased disease resistance was also observed among alfalfa, *Arabidopsis*, and cotton (Gallego-Giraldo et al. 2011, 2020; Hu et al. 2018). Thus, the relationship between the total lignin content and *V. dahliae* resistance is yet to be further investigated using more cotton accessions with different levels of *V. dahliae* resistance.

Silencing *Gh4CL30* alters lignin biosynthesis.

WGCNA is an effective way to identify candidate hub genes and has been successfully used in strawberry (Hollender et al. 2014), soybean (Du et al. 2017), rice (Tan et al. 2017), and cotton (Sun et al. 2019). In this study, we identified several candidate hub genes associated with *V. dahliae* resistance based on WGCNA, including *Gh4CL30* that is related to lignin biosynthesis. Several *4CLs* genes have been reported to be responsive to pathogen stresses (Ehlting et al. 1999; Oliveira et al. 2015; Sun et al. 2013; Xu et al. 2011). In *Arabidopsis*, upon *Peronospora parasitica* infection, the expression of *At4CL1* and *At4CL2* was strongly increased at 12 and 24 hpi (Ehlting et al. 1999). *Pv4CL* was upregulated in dry bean plants infected by *Sclerotinia sclerotiorum* (Oliveira et al. 2015). Similarly, *Gb4CL* was induced by *V. dahliae* infection in *G. barbadense* (Sun et al. 2013; Xu et al. 2011). Following *V. dahliae* inoculation, we observed a consistently significant increase of *Gh4CL30* in the roots and stems of the two cotton cultivars used in this study (Fig. 5B and C). *Gh4CL30* is related to lignin biosynthesis and a hub gene of the network related to disease response in HR and HS; thus, we further characterized its function in *V. dahliae* resistance. Knockdown of the expression of *Gh4CL30* by VIGS led to enhanced resistance to *V. dahliae* in HS (Fig. 6), suggesting a negative regulatory role of *Gh4CL30* in *V. dahliae* resistance. Similarly, a *4CL*-like gene, *OsAAE3*, has been shown to negatively mediate blast resistance in rice (Liu et al. 2017).

Phylogenetic analysis revealed that *Gh4CL30* is more closely related to *Arabidopsis At4CL1* and *At4CL2* than to *At4CL3* and *At4CL4* (Feng et al. 2017), suggesting a function of *Gh4CL30* similar to that of *At4CL1* and *At4CL2*, which contribute to lignin biosynthesis in the xylem (Li et al. 2015). As the key gene in lignin biosynthesis, the function of *4CL* in lignin biosynthesis has been extensively investigated in *Arabidopsis*, rice, and poplar (Ehlting et al. 1999; Gui et al. 2011; Li et al. 2015; Voelker et al. 2010). Similar to what has been found in these plants, downregulation of *Gh4CL30* resulted in a decreased level of lignin (Fig. 7A and B), most likely due to a down-regulated level of lignin biosynthesis-related genes (Fig. 7C to H); however, it is unexpected that we did not observe decreased disease resistance in the *Gh4CL30*-silenced plants (Fig. 6). It has been suggested that both the contents of the G and S monomers and the G/S ratio could be correlated to disease resistance (Gayoso et al. 2010; Xu et al. 2011). The G/S ratio was increased and decreased in *V. dahliae*-resistant and -susceptible cotton varieties, respectively (Gayoso et al. 2010; Xu et al. 2011). Consistently, we found that the G/S ratio was lower in

the *V. dahliae*-infected *TRV:00* and HS plants than in the uninfected *TRV:00* and HS plants (Fig. 3F; Table 2). However, in the *Gh4CL30*-silenced and HR plants, an increased G/S ratio was observed after *V. dahliae* infection (Fig. 3F; Table 2), similar to what has been observed in the *V. dahliae*-resistant cotton (Gayoso et al. 2010; Xu et al. 2011). This suggests that the G/S ratio rather than the total lignin content determines *V. dahliae* resistance in cotton and explains why plants with a decreased level of total lignin level showed enhanced *V. dahliae* resistance.

Silencing of *Gh4CL30* represses the SA and JA biosynthesis or signaling pathways and flavonoid biosynthesis.

In *Medicago sativa*, downregulation of *HCT* led to decreased lignin content and increased accumulation of SA and JA, and enhanced resistance to *Colletotrichum trifolii* (Gallego-Giraldo et al. 2011). Methyl jasmonate and SA regulate the expression level of *4CL* (Gao et al. 2015; Lee and Douglas 1996), and both SA and JA have been shown to play a major role in the cotton-*V. dahliae* interaction (Shaban et al. 2018). We were interested to know whether *Gh4CL30* regulates *V. dahliae* resistance through the SA and JA biosynthesis or signaling pathways. We found that silencing *Gh4CL30* reduced SA content in both uninfected and infected plants but reduced JA content only in the uninfected plants (Supplementary Fig. S8A and B), suggesting that *Gh4CL30*-silencing-induced reduction of JA biosynthesis could be compensated for by other pathways induced by *V. dahliae* infection, and that the SA and JA biosynthesis pathways may not be the major contributors of the improved *V. dahliae* resistance observed in *Gh4CL30*-silenced plants.

4CL has also been shown to be important in the biosynthesis of flavonoids (Lavhale et al. 2018). The role of flavonoids on plant-pathogen interactions, by which they can inhibit the growth of pathogens, is well documented (Dixon and Steele 1999; Gill et al. 2018). A recent study in cotton showed that silencing a lignin biosynthesis-related gene, *GhLAC1*, results in decreased total lignin content while increasing accumulated flavonoids and enhanced plant resistance to *V. dahliae* (Hu et al. 2018). We found that the *Gh4CL30*-silenced plants produced fewer total flavonoids compared with the *TRV:00* plants but the methanolic extracts from the stems of the *Gh4CL30*-silenced plants could significantly inhibit *V. dahliae* hyphal growth (Fig. 8A and B; Supplementary Fig. S9A and B), suggesting that *Gh4CL30* may positively regulate biosynthesis of flavonoids, which is different from what has been reported for the *4cl1* mutant in *Arabidopsis* (Li et al. 2015); however, flavonoids may not be the metabolites contributing to the enhanced *V. dahliae* resistance observed in the *Gh4CL30*-silenced plants.

Accumulation of phenolics induced by silencing of *Gh4CL30* contributes to increased cotton resistance to *V. dahliae*.

It is well known that deposition of phenolics acts as an important physical and biochemical barrier contributing to plant defense against vascular pathogens (Eynck et al. 2009; Xu et al. 2011). Downregulating the expression of *4CL* could lead to the accumulation of soluble phenolic compounds that are toxic to some pathogens (Miedes et al. 2014). Similar to the results reported in studies on the effects of downregulation of *4CL1* in several other species (Voelker et al. 2010; Zhou et al. 2015), we observed red-brown xylem and increased content of phenolics in the *Gh4CL30*-silenced plants (Supplementary Figs. S6 and S7; Fig. 8C and D). Cinnamic acid, coumalic acid, caffeic acid, and ferulic acid are the four major phenolics catalyzed by *4CL* for monolignin biosynthesis in higher plants, and they have antifungal and antibacterial functions (Boutigny et al. 2009;

Lanoue et al. 2010; Lavhale et al. 2018). Our previous study found that *Gh4CL30* exhibited activity toward cinnamic acid, coumalic acid, caffeic acid, and ferulic acid, with the preferred substrate being ferulic acid (Feng et al. 2017). The abundance of caffeic acid and ferulic acid was significantly increased while that of cinnamic acid and coumalic acid was unchanged in stems of the *Gh4CL30*-silenced plants compared with that of *TRV:00* (Fig. 8C and D), suggesting that caffeic acid and ferulic acid might be the prominent substances of *Gh4CL30*. We further demonstrated the role of caffeic acid and ferulic acid in inhibition of *V. dahliae* hyphal growth (Fig. 8G and H), consistent with the results observed in other studies (Boutigny et al. 2009; Dimkić et al. 2016; Dixon 2001). These results indicated that accumulation of caffeic acid and ferulic acid caused by silencing of *Gh4CL30* is responsible for the increased *V. dahliae* resistance of the *Gh4CL30*-silenced plants.

MATERIALS AND METHODS

Plant materials and culture conditions.

V. dahliae-resistant *G. hirsutum* ‘Shidalukang 1’ (HR) and *V. dahliae*-susceptible *G. hirsutum* ‘Junmian 1’ (HS) were provided by the Cotton Research Institute of Shihezi University and used for RNA-seq and VIGS experiments (Liu et al. 2002). Cotton seed were sterilized with 0.1% (wt/vol) HgCl_2 for 10 min, then rinsed three times with sterilized distilled water. Cotton seedlings used for all the experiments were grown in a greenhouse under conditions of 24 and 22°C day and night temperatures, respectively; a photoperiod of 16 h of light and 8 h of darkness; 50% relative humidity; and average daylight intensity of $240 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Pathogenicity assays.

V991, a highly aggressive defoliating *V. dahliae* strain, was kindly provided by Associate Professor Yanfei Sun (Shihezi University). The fungi were subcultured on potato dextrose agar (PDA) medium at 25°C in darkness for 7 days. Colonies were then inoculated into Czapek medium for 7 days at 25°C with shaking (150 rpm). Two-leaf-stage seedlings were inoculated with 10 ml of spore suspensions (10^6 conidia ml^{-1}) by the root irrigation method (Xiong et al. 2019). Seedlings inoculated with sterile water were used as mock controls. After inoculation, plants were transferred to a greenhouse. Samples were harvested at 0, 12, 24, and 48 hpi from five cotton plants. For each sample, a mixture of 200 mg of roots and 200 mg of stems was used in RNA extraction and RNA-seq. Three biological replicates were sampled for each combination of genotype and time point.

RNA extraction, library construction, and sequencing.

Twenty-four samples (four time points by two cultivars by three biological replicates) from HR and HS were used for RNA-seq. Total RNA was isolated from each sample using a plant RNA Purification Kit (Tiangen, Beijing) according to the manufacturer’s instructions. RNA integrity and purity were assessed using the RNA Nano 6000 Assay Kit with the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, U.S.A.) and a NanoPhotometer spectrophotometer (Implen, Westlake Village, CA, U.S.A.), respectively. A total amount of 3 μg of RNA per sample was used in preparation of RNA-seq libraries that were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, U.S.A.) following the manufacturer’s recommendations. RNA-seq (150-bp paired-end reads) was performed by Novogene (Novogene, Tianjin, China) on an Illumina HiSeq 4000 platform. The raw RNA-seq data are available in the NCBI under accession number PRJNA593765.

Identification and functional annotation of DEGs.

Clean reads were generated using in-house Perl scripts by removing reads containing adapter or ploy-N as well as with a phred quality score < 20 at each nucleotide from the raw data. Paired-end clean reads were aligned to the *G. hirsutum* reference genome (Zhang et al. 2015) using TopHat v2.0.12.

Gene expression levels were determined by fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) (Trapnell et al. 2010). Differentially expressed genes were identified by the DESeq R package (1.18.0) (Wang et al. 2010) using the criterion of an adjusted *P* value < 0.05 (Anders and Huber 2010). DEGs were subjected to GO enrichment analysis by Blast2GO with a false discovery rate < 0.05 (Young et al. 2010). Additionally, KEGG pathways of DEGs were tested for statistical enrichment using the KOBAS 2.0 software with a *P* value < 0.05 (Kanehisa et al. 2008). The Venn diagram and heat map were generated by the Novemagic server.

WGCNA analysis.

Coexpression networks were constructed as described previously using the WGCNA (v1.29) package in R (Langfelder and Horvath 2008). The analysis was based on the FPKM of the 6,831 DEGs. The modules were generated with the default settings, except that the power was set to 14, TOMType was signed, minModuleSize was 30, and merge CutHeight was 0.25. Each module was represented by a different color.

RT-qPCR analysis.

cDNA was synthesized from 3 μg of total RNA using the M-MLV reverse transcription (TaKaRa, Dalian, China). RT-qPCR analyses were conducted using the SYBR Green (Roche, Rotkreuz, Switzerland) on Light Cycler 480II (Roche). All primers used in RT-qPCR were designed using Primer3 Plus and are listed in Supplementary Table S1. The cotton *UBQ7* gene (DQ116441.1) was used as an internal control to normalize the variance among samples. PCR conditions consisted of an initial denaturation step at 95°C for 5 min and 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 20 s. The relative expression level from three biologically independent experiments was evaluated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001).

VIGS experiments.

Tobacco rattle virus (TRV) vectors were used for VIGS experiments. *GhCHLI* (encoding magnesium chelatase subunit I) was used as a positive control to check the silencing efficiency. The TRV empty vector (*TRV:00*) was employed as the negative control. Approximately 400-bp fragments were amplified from *Gh4CL30* and *GhCHLI* using the primers listed in Supplementary Table S1 and were then used to construct *TRV:Gh4CL30* and *TRV:GhCHLI* vectors, respectively. The TRV-mediated gene silencing in cotton was performed as described previously (Gao et al. 2013). RNA was extracted from cotton stems to measure target gene expression when *TRV:GhCHLI* cotton plants exhibited yellowing, approximately 10 days after TRV infection.

Characterization of *V. dahliae* resistance in VIGS cotton.

Two-leaf-stage *TRV:Gh4CL30* and *TRV:00* cotton plants were inoculated with 10-ml *V. dahliae* spore suspensions (10^6 conidia ml^{-1}) by the root irrigation method (Xiong et al. 2019). At least 30 cotton seedlings of similar size were used in each treatment. The ratio of diseased plants and disease index were investigated at 14 and 21 dpi. To compare the vascular phenotype, the stems above cotyledons of the infected seedlings were cut at 14 dpi and observed by using a stereo microscope (Stereo Discovery.V20; Zeiss). Fungal biomass quantification

and fungal recovery assays were performed as previously described (Atallah et al. 2007). Briefly, total DNA was isolated from stems of the infected cotton plants at 14 dpi. Primers for the cotton *UBQ7* gene were used to quantify cotton DNA, and *V. dahliae*-specific primers ITS1-F and ST-Ve1-R were used to quantify fungal DNA (Supplementary Table S1) (Ellendorff et al. 2009). For stem culture, the stem section above the cotyledons was collected from 10 infected plants at 14 dpi. The sections were surface sterilized in 3% hypochlorite for 3 min and rinsed three times with sterile water, then cut into 1-cm slices and plated on PDA medium at 25°C in darkness.

Lignin histochemical staining.

Visualization of histochemical staining of lignin was done using the Wiesner staining method (Xu et al. 2011). At 48 hpi, stems at the same position were collected from mock- and *V. dahliae*-inoculated HR, HS, *TRV:00*, and *TRV:Gh4CL30* plants, hand cut into slices, and dipped in a phloroglucinol solution (2% [vol/vol] in 95% ethanol) for 5 min, followed by immersion in 18% hydrochloric acid for 5 min, then fixed on slides to be observed and photographed under a fluorescence microscope (SterEO Discovery.V20; Zeiss) immediately. Red color indicated the presence of lignin.

Determination of total lignin content and lignin monomer.

The total lignin content of stem tissue was determined by the acetyl bromide method as described previously, with minor modifications (Fukushima and Hatfield 2004). Briefly, stem samples (approximately 100 mg; the precise weight was recorded) were quickly ground to powder in liquid nitrogen, then successively washed with ethanol, 95% ethanol, and deionized water to remove soluble substances. After freeze drying, 5 ml of acetyl bromide-glacial acetic acid (1:3, vol/vol) was added into each sample and incubated at 70°C for 30 min. Then, 0.9 ml of 2 M NaOH and 3 ml of glacial acetic acid were added, and finally supplied with 1 ml of 7.5 M hydroxylamine hydrochloride solution to terminate the reaction. After centrifugation at 13,000 rpm, the supernatant was collected, and the absorbance at 280 nm was measured with a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, U.S.A.). Alkali lignin was used as a standard.

The lignin monomer composition of stems was determined for three biological replicates by the thioacidolysis and gas-chromatography mass-spectrometry method (Zhao et al. 2010). The lignin monomer composition was expressed as the percentage of dry weight.

Determination of the total content of flavonoids.

The total flavonoid content was measured following the AlCl₃ colorimetric assay as previously described, with slight modifications (Dewanto et al. 2002). Briefly, at 48 hpi, stems were collected from mock- and *V. dahliae*-inoculated *TRV:00* and *TRV:Gh4CL30* plants. Stems were ground into powder in liquid nitrogen, and 100 mg of frozen powder was dissolved with 2 ml of extraction solvent (80% methanol). After centrifugation at 13,000 rpm, 30 µl of 5% NaNO₂ (wt/vol) solution was added to the supernatant and incubated at room temperature. After 5 min of incubation, 60 µl of 10% (wt/vol) AlCl₃ solution was added. Samples were well mixed and incubated at room temperature for 5 min. Finally, 0.2 ml of 1 M NaOH solution was added to each sample. The absorbance at 500 nm was immediately measured with a NanoDrop ND-2000 spectrophotometer (Thermo Scientific). Rutin was used as a standard.

Extraction and quantification of phytohormone content.

The extraction and measurement of the endogenous SA and JA were performed as described by Sun et al. (2014), with slight

modifications. Approximately 200 mg of fresh stem from *TRV:00* and *TRV:Gh4CL30* plants was ground to a fine powder in liquid nitrogen and extracted twice with 2 ml of precooled 80% methanol overnight in the dark at 4°C. The supernatant was filtered with a 0.22-mm filter membrane and dried under N₂ at room temperature, then dissolved in 0.4 ml of methanol. The JA and SA levels were quantified using the high-performance liquid chromatography-tandem mass spectrometry (LC/MS) system (AB SCIEX Triple Quad 5500) with JA and SA (Sigma-Aldrich, St. Louis) as the internal standards.

Analyses of the contents of four phenolic compounds.

To determinate the endogenous concentration of caffeic acid, coumalic acid, ferulic acid, and cinnamic acid, stem samples from mock- and *V. dahliae*-inoculated *TRV:00* and *TRV:Gh4CL30* plants 48 hpi were dried in a lyophilizer and ground to a fine powder. The powder (0.5 g) was extracted twice with 1 ml of 80% methanol followed by sonication for 1 h at 60°C and extracted at 4°C overnight. The samples were centrifuged at 12,000 rpm for 5 min. The supernatant was collected into a fresh tube and dried with nitrogen gas at room temperature to a final volume of approximately 1 ml. After filtering through a 0.45-µm membrane filter, the extract was analyzed for the concentrations of phenolics on LC/MS according to a previous study (Proestos and Komaitis 2013).

AUTHOR-RECOMMENDED INTERNET RESOURCES

Novemagic server: <https://magic.novogene.com/customer/main#/login>
Primer3 Plus: <http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>

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