The Cotton Wall-Associated Kinase GhWAK7A Mediates Responses to Fungal Wilt Pathogens by Complexing with the Chitin Sensory Receptors

Ping Wang, a,b Lin Zhou, c Pierce Jamieson, a Lin Zhang, c Zhixue Zhao, a Kevin Babila, c Wenyong Shao, c,d Lizhu Wu, c,d Roma Mustafa, c,e Imran Amin, e Alessandra Diomaiuti, f Daniela Pontiggia, f Simone Ferrari, f Yuxia Hou, b Ping He, c and Libo Shan a,b,1

a Department of Plant Pathology and Microbiology, and Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, Texas 77843
b College of Science, China Agricultural University, Beijing 100193, China
c Department of Biochemistry and Biophysics, and Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, Texas 77843
d North China Key Laboratory for Germplasm Resources of Education Ministry, Hebei Agricultural University, Baoding, Hebei 071001, China
e Agricultural Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Faisalabad, Punjab, 44000 Pakistan
f Università di Roma Sapienza, Dipartimento di Biologia e Biotecnologie “Charles Darwin,” 00185 Roma, Italy

ORCID IDs: 0000-0002-4881-2804 (P.W.); 0000-0002-1010-5610 (L.Z.); 0000-0001-5929-5534 (P.J.); 0000-0002-5070-7667 (L.Z.); 0000-0002-3450-8422 (Z.X.Z.); 0000-0002-2980-4526 (K.B.); 0000-0002-1824-2302 (W.Y.S.); 0000-0002-9324-6143 (L.H.W.); 0000-0002-8979-9044 (R.M.); 0000-0003-3063-4103 (I.A.); 0000-0002-3758-3743 (A.D.); 0000-0002-1389-2090 (S.F.); 0000-0003-2975-9953 (Y.X.H.); 0000-0002-5926-8349 (P.H.); 0000-0003-3063-4103 (I.A.); 0000-0002-4798-9907 (L.S.);

Plant receptor-like kinases (RLKs) are important players in response to pathogen infections. Verticillium and Fusarium wilts, caused by Verticillium dahliae (Vd) and Fusarium oxysporum f. sp vasinfectum (Fov), respectively, are among the most devastating diseases in cotton (Gossypium spp). To understand the cotton response to these soil-borne fungal pathogens, we performed a genome-wide in silico characterization and functional screen of diverse RLKs for their involvement in cotton wilt diseases. We identified Gossypium hirsutum GhWAK7A, a wall-associated kinase, that positively regulates cotton response to both Vd and Fov infections. Chitin, the major constituent of the fungal cell wall, is perceived by lysin-motif-containing RLKs (LYKs/CERK1), leading to the activation of plant defense against fungal pathogens. A conserved chitin sensing and signaling system is present in cotton, including chitin-induced GhLYK5-GhCERK1 dimerization and phosphorylation, and contributes to cotton defense against Vd and Fov. Importantly, GhWAK7A directly interacts with both GhLYK5 and GhCERK1 and promotes chitin-induced GhLYK5-GhCERK1 dimerization. GhWAK7A phosphorylates GhLYK5, which itself does not have kinase activity, but requires phosphorylation for its function. Consequently, GhWAK7A plays a crucial role in chitin-induced responses. Thus, our data reveal GhWAK7A as an important component in cotton response to fungal wilt pathogens by complexing with the chitin receptors.

INTRODUCTION

In addition to its use in textile manufacturing, cotton (Gossypium spp) serves as an agro-economically important source of feed, foodstuff, oil, and biofuel. Within the genus Gossypium, there are 41 to 47 diploid species grouped into eight diploid genome groups (A through G, and K) and seven allotetraploid species, which were formed likely from interspecific hybridizations between the A-genome species resembling G. herbaceum or G. arboreum and the D-genome species G. raimondii (Zhang et al., 2008; Wang et al., 2018; Hu et al., 2019). After the polyploidization event, the allotetraploids gave rise to three modern lineages, including the agronomically important G. hirsutum and G. barbadense (Zhang et al., 2008; Wang et al., 2018). G. hirsutum, known as upland cotton, is the most widely cultivated species of cotton and constitutes ~90% of cotton output worldwide. The genome sequences of G. raimondii (D2), G. arboreum (A2), and G. hirsutum (AD1) have been released (Paterson et al., 2012; Wang et al., 2012; Li et al., 2014, 2015; Yuan et al., 2015), which provides the foundations to understand the cotton genome organization, genetic variations, and speciation processes. Moreover, these resources facilitate functional genomic studies, which provide key information for improving the agricultural performance of cotton in the face of biotic and abiotic stresses by the manipulation of cotton genes and their regulation. Verticillium wilt caused by Verticillium dahliae Kleb (Vd) and Fusarium wilt caused by Fusarium oxysporum f. sp vasinfectum...
(Fov) are among the destructive cotton vascular diseases faced by growers. Both diseases show symptoms such as foliar chlorosis or necrosis, premature leaf defoliation, vascular discoloration, plant stunting, and wilt (Li et al., 2017b; Cox et al., 2019). Due to the vitality of resting spores and overwintering structures produced by Fov (chlamydospores) and Vd (microsclerotia), they are notoriously difficult to eradicate from infected fields (Wheeler et al., 2012; Cox et al., 2019). Currently, the development and application of disease-resistant cultivars are among the effective means to control these pathogen threats (Cianchetta and Davis, 2015; Sanogo and Zhang, 2016). The biosynthesis of phenylpropanoids, terpenoids, and reactive oxygen species (ROS), along with the salicylic and jasmonic acid signaling pathways, contribute to cotton defense against Vd (Gao et al., 2013a; Ashraf et al., 2018; Hu et al., 2018). Vd and Fov infection likely share overlapping and distinct features, but the molecular mechanisms of Fov infection and the determinants of Fov resistance in cotton remain largely elusive.

At the cellular level, the first line of plant innate immunity, known as pattern-triggered immunity (PTI), is elicited through perception of microbe-associated molecular patterns (MAMPs) by plant cell surface-resident pattern recognition receptors (Yu et al., 2017). Several pattern recognition receptors have been identified as receptor-like kinases (RLKs) or receptor-like proteins (RLPs; Böhm et al., 2014; Couto and Zipfel, 2016). Both RLKs and RLPs contain an extracellular domain and a single transmembrane domain, but RLPs lack the cytoplasmic kinase domain found in RLKs (Shiu and Bleecker, 2003; Jamieson et al., 2018). The extracellular domains vary in RLKs and RLPs, including the leucine-rich repeat (LRR), the lectin motif, the lysin motif (LysM), and the epidermal growth factor (EGF)-like motif (Böhm et al., 2014; Couto and Zipfel, 2016).

Emerging evidence has suggested that RLKs and RLPs play an essential role in resistance to Verticillium and Fusarium wilt diseases in a range of plant species. The most common extracellular domain of RLKs and RLPs is LRR. The tomato (Solanum lycopersicum) LRR-RLP Ve1 confers race-specific resistance to Vd carrying Ave1 (Kawchuk et al., 2001). Ve1 homologs in cotton play a role in Verticillium wilt resistance to certain isolates (Gao et al., 2011; Zhang et al., 2012). Further, in tomato, Ve1 mediates resistance to F. oxysporum f. sp lycopersici carrying Ave1 (de Jonge et al., 2012). LRR-RLPs I-1 and I-7 confer resistance to F. oxysporum f. sp lycopersici in tomato (Huang et al., 1997; Houterman et al., 2008; Gonzalez-Cendales et al., 2016).

LysM-containing RLKs and RLPs are important players during plant-fungal interactions by mediating chitin recognition and signaling activation (Wan et al., 2008; Tanaka et al., 2013; Gong et al., 2020). Chitin, the polymer of β-1,4-linked N-acetylglucosamine, is a component of the fungal cell wall and known as a potent MAMP in many plant species. In rice (Oryza sativa), OsCEBiP, an LysM-RLP, functions as a high-affinity chitin binding receptor and homodimerizes to sandwich chitin in a receptor complex at the plasma membrane (Kaku et al., 2006; Hayafune et al., 2014). OsCERK1, a LysM-RLK, which lacks chitin binding activity, associates with OsCEBiP and is essential for signaling activation (Shimizu et al., 2010). There are five LysM-RLKs (LYKs) and three LysM-RLPs (LYPs/LYM s) in the Arabidopsis (Arabidopsis thaliana) genome. AtCERK1 (AtLYK1), AtLYK4, and AtLYK5 are important for chitin perception and signaling (Miya et al., 2007; Liu et al., 2012; Wan et al., 2012; Cao et al., 2014). AtLYK5 possesses higher binding affinity to chitin than AtCERK1, and chitin perception induces AtLYK4/AtLYK5-AtCERK1 complex formation and AtLYK5-mediated AtCERK1 phosphorylation (Cao et al., 2014; Xue et al., 2019). AtLYM2 regulates chitin-induced stomatal closure in Arabidopsis (Faulkner et al., 2013). Distinct chitin perception and signaling systems exist in Arabidopsis and rice (Wan et al., 2012; Kawasaki et al., 2017).

Wall-associated kinases (WAKs) are a unique group of RLKs with extracellular EGF motifs, which likely link to the cell wall through the homogalacturonan binding domain (Decreux et al., 2006; Kanneganti and Gupta, 2008). The five Arabidopsis AtWAKs are clustered in a 30-kb locus on chromosome 1 with high sequence similarity (>85% identical for the kinase domains; He et al., 1999). AtWAKs are involved in cell expansion during plant development (Wagner and Kohorn, 2001). AtWAK1 was shown to bind to both long pectin molecules resident in native cell walls and short oligogalacturonic acid fragments released during pathogen infection or wounding in vitro (Decreux and Messiaen, 2005). Similarly, AtWAK2 is able to bind to the de-esterified charged galacturonic acid backbone common to pectin (Kohorn et al., 2009). Besides, AtWAK1 was proposed to be a receptor of oligogalacturonides (OGs), which are released from the plant cell wall during pathogen infections and is required for the resistance to Botrytis cinerea (Brutus et al., 2010). In recent years, several independent studies have highlighted the importance of the WAK gene family in resistance against pathogen infections in crops. Two WAK genes from maize (Zea mays) were discovered as quantitative resistance loci important for resistance against northern maize leaf blight and head smut (Hurni et al., 2015; Zuo et al., 2015; Yang et al., 2019). The disease resistance gene Xanthomonas oryzae pv oryzae resistance 4 in rice encodes a WAK that promotes cellulose synthesis and strengthens the plant cell wall, thereby enhancing plant resistance against both bacterial (X. oryzae pv oryzae) infection and lodging (Hu et al., 2017). SIWAK1 from tomato promotes the apoplast immunity against bacterial pathogen Pseudomonas syringae (Zhang et al., 2020). Besides, the Arabidopsis genome contains 21 WAK-like (WAKLs) genes (Verica and He, 2002; Verica et al., 2003), some of which play a role in plant disease resistance. For example, Arabidopsis WAKL RFO1, and LRR-RLP RFO2 confer quantitative resistance to a broad spectrum of different Fusarium races, suggesting that a common elicitor or MAMP in Fusarium might contribute to the resistance (Diener and Ausubel, 2005; Shen and Diener, 2013). Thus, both WAK and LYK family genes are important regulators in plant resistance against different fungal pathogens.

To test whether the WAK and LYK family genes play a role in cotton fungal disease resistance, we performed a genome-wide identification of cotton WAK and LYK family genes in silico and identified 13 GhWAK and 17 GhLYK genes in G. hirsutum. The phylogenetic analysis unraveled the evolutionary conservation of LYKs, and the interspecies divergence of WAKs between cotton and Arabidopsis. Together with RNA sequencing (RNA-seq), loss-of-function screens using Agrobacterium (Agrobacterium tumefaciens) mediated virus-induced gene silencing (VIGS) and biochemical assays, GhWAK7A, GhCERK1, and GhLYK5 were demonstrated to contribute to cotton resistance to Fov and Vd and...
mediate chitin perception and signaling in cotton. GhWAK7A phosphorylates GhLYK5 and regulates chitin-induced GhCERK1 and GhLYK5 association. Our results reveal that a wall-associated RLK, GhWAK7A, functions in cotton fungal disease resistance via the modulation of chitin signaling.

RESULTS

Identification of WAK Family Genes in Cotton

WAKs represent a class of RLKs characterized by an aminol (N)-terminal conserved signal peptide, an extracellular WAK galacturonic binding domain, an EGF-like domain, an EGF calcium binding (EGF-Ca\(^{2+}\)) domain, a transmembrane domain, and a carboxyl (C)-terminal cytosolic Ser/Thr protein kinase domain (Verica and He, 2002). The Arabidopsis genome encodes five WAKs and 21 WAKL genes, which bear different extracellular domain configurations and show only 18% to 22% identities in their extracellular regions to WAKs (Verica et al., 2003). Our study here is mainly focused on the genome-wide identification of cotton WAK genes. Using five Arabidopsis AtWAK proteins as queries, we performed a genome-wide identification of cotton WAK genes in the genome database of representative cotton species including an allotetraploid cotton (G. hirsutum) and two diploid cotton species (G. arboreum and G. raimondii). The databases used in this study included the Nanjing Agricultural University (NAU) assembly for G. hirsutum (Zhang et al., 2015), the Joint Genome Institute (JGI) assembly for G. raimondii (Paterson et al., 2012), and the Cotton Research Institute (CRI) assembly for G. arboreum (Li et al., 2014).

To identify WAK genes in these cotton species, we implemented a pipeline wherein BLASTp (https://cottonfgd.org/blast/) search results were refined manually by evaluating protein domain predictions for subject amino acid sequences (Figure 1A). First, the full-length amino acid sequences of five AtWAKs were used as the queries for the BLASTp search against the three cotton genome databases G. hirsutum (AD)-NAU, G. raimondii (Dj)-JGI, and G. arboreum (A)-CRI, with a threshold of E-value < 1e-50. Second, proteins annotated as non-WAK proteins or WAKL genes were removed from the list. Furthermore, we verified the presence of the conserved above-mentioned various WAK domains of candidates. The domain prediction was performed using the protein sequence analysis tools from the programs SMART (http://smart.embl-heidelberg.de/) and Interpro (https://www.ebi.ac.uk/interpro/). Proteins, which lack essential domains of WAKs, were eliminated from the data set. After manually evaluating the protein predictions of each candidate, we identified 13 putative GhWAKs from the G. hirsutum (Supplemental Table 1), 16 GaWAKs from the G. arboreum (Supplemental Table 2), and 11 GrWAKs from the G. raimondii (Supplemental Table 3). G. arboreum has more WAK members than G. hirsutum, suggesting massive gene deletions during tetraploid G. hirsutum speciation (see below for examples). The phylogenetic analysis of these candidates with AtWAKs and AtWAKLs revealed that they are phylogenetically closer to AtWAKs than AtWAKLs, indicating that the cotton proteins we identified here are WAKs, but not WAKLs (Supplemental Figure 1).

Phylogenetic Analysis and Evolution of WAK Family Genes in Cotton

To elucidate the evolutionary relationship of WAK family proteins in Arabidopsis and cotton, we performed a phylogenetic analysis of cotton and Arabidopsis WAK proteins using the tool MEGA X (Kumar et al., 2018). No cotton WAKs from G. hirsutum, G. arboreum, and G. raimondii were found to interleave with the isolated clade formed by the five Arabidopsis AtWAKs (Supplemental Figure 2). This result remains consistent when the WAKs from G. hirsutum, G. arboreum, or G. raimondii database were analyzed separately (Figure 1B and Supplemental Figures 3A and 3B). The phylogeny using the kinase domains also indicated that WAKs from different cotton species form a separate clade from AtWAKs (Supplemental Figure 4). The five AtWAK proteins share 60% to 80% identities while the identities among AtWAK and GhWAK proteins are 30% to 50% (Supplemental Table 4). Collectively, this suggests that members of WAKs in cotton and Arabidopsis have likely evolved independently. Based on the phylogenetic relationships, we divided the cotton WAK members into five clades (I to V) with GhWAK1D, GhWAK2D, and GhWAK3D in clade I; GhWAK4D in clade II; GhWAK5A and GhWAK5D in clade III; GhWAK6D, GhWAK7A, GhWAK8A, and GhWAK8D in clade IV; and GhWAK9D, GhWAK10A, and GhWAK10D in clade V in G. hirsutum (Figure 1B; Supplemental Figure 2; Supplemental Table 5). Because this study is focused on GhWAKs, we named the individual members of WAKs from G. hirsutum. In consideration of the allotetraploid nature of G. hirsutum (AADD), the names of GhWAKs were followed with an uppercase “A” or “D” indicating the gene derived from the A- or D-subgenome (Figure 1B; Supplemental Table 6). For the homoeologous genes in A- and D-subgenomes, we gave the same name followed by an “A” or “D,” such as GhWAK10A and GhWAK10D.

Among 13 GhWAKs, nine are in the D-subgenome and four are in the A-subgenome (Figure 1C). The phylogenic analysis showed all the four GhWAK genes from the A-subgenome are orthologous to genes from the G. arboreum genome (Supplemental Figures 5A and 6) and three of them have corresponding D-subgenome homologs (Figure 1B, Supplemental Figure 6). Interestingly, three GhWAKs (GhWAK7A, GhWAK8A, and GhWAK10A) on chromosome 2 of G. hirsutum A-subgenome are orthologous to WAKs on chromosome 3 of G. arboreum A-genome (Figure 1C; Supplemental Figure 5A), which may be due to the reciprocal translocation during the speciation of tetraploid G. hirsutum (Hu et al., 2019). Apparently, 12 out of 16 GhWAKs from G. arboreum were lost during G. hirsutum speciation (Supplemental Figures 5A and 6). Of the nine GhWAKs from the D-subgenome, seven genes have corresponding orthologs from G. raimondii (Supplemental Figures 5B and 6). Two other genes, GhWAK1D and GhWAK3D, are tandemly arranged and share high protein identity with GhWAK2D (92% and 87% respectively; Figure 1C; Supplemental Table 4), indicating they might have recently evolved as a result of tandem gene duplication in G. hirsutum. One pair of orthologs in G. raimondii and G. arboreum (Ga03G08241.1; Gori.005G086200.1) do not have associated G. hirsutum counterparts (Supplemental Figure 6), suggesting that either the G. hirsutum ortholog was lost during the speciation, or the G. hirsutum ortholog was not able to be resolved with the genome assembly used in this study.
We attempted to further understand how WAK genes have evolved in cotton by comparing the number and relatedness of WAKs in *A. thaliana*, *G. arboreum*, *G. raimondii*, and *G. hirsutum*. Inferences made using the phylogenetic analysis between GhWAKs from the A-subgenome and GaWAKs, and GhWAKs from the D-subgenome and GrWAKs (Supplemental Figures 5A, 5B, and 5C, and 6), suggested that the trajectory of WAK gene number changes during the cotton genome evolution (Figure 1D). We propose that there might be seven WAK genes in the most recent common ancestor of two diploid cotton species (*G. arboreum* and *G. raimondii*) with massive gene duplications and losses during the evolution of *G. arboreum*, *G. hirsutum*, *G. arboreum*, and *G. raimondii*. The phylogenetic tree was generated by the neighbor-joining method with 1,000 bootstrap replicates in the program MEGA X. The five AtWAKs are shown in blue. The NAU accession ID of each GhWAK is labeled adjacent to the GhWAK name curated in this study. Gene structures are further diagrammed next to each GhWAK using purple boxes to indicate exons and black shrunken lines with the same length to indicate introns. Schematics depicting WAK protein motifs were manually confirmed using the tool SMART (http://smart.embl-heidelberg.de/). A legend for the protein domain schematics is included at the bottom. Alignments corresponding to these analyses can be found in Supplemental Files 1 and 2.

**Figure 1.** Identification and Phylogenetic Analysis of WAK Family Proteins in Cotton.

(A) Genome-wide identification pipeline of WAKs in cotton genomes. Five Arabidopsis WAK genes annotated in TAIR10 were used as the query sequences for a BLASTp (cutoff: E-value < 1.0e-50) against three cotton genomes. The results from this BLAST search were refined using protein domain prediction tools (e.g., SMART, InterPro) to eliminate non-WAKs by manually screening proteins without any conserved WAK motifs.

(B) A phylogenetic view of GhWAK family proteins, gene structures, protein motifs, and clade partitions. The five AtWAK proteins were included to depict the evolutionary distance of the WAK proteins between *G. hirsutum* and *A. thaliana*. The phylogenetic tree was generated by the neighbor-joining method with 1,000 bootstrap replicates in the program MEGA X. The five AtWAKs are shown in blue. The NAU accession ID of each GhWAK is labeled adjacent to the GhWAK name curated in this study. Gene structures are further diagrammed next to each GhWAK using purple boxes to indicate exons and black shrunken lines with the same length to indicate introns. Schematics depicting WAK protein motifs were manually confirmed using the tool SMART (http://smart.embl-heidelberg.de/). A legend for the protein domain schematics is included at the bottom. Alignments corresponding to these analyses can be found in Supplemental Files 1 and 2.

(C) Genomic locations of GhWAK genes on their corresponding chromosomes. White bars represent chromosomes from the A-subgenome of *G. hirsutum* while gray bars represent the D-subgenome chromosomes. Chromosomes that were not found to contain WAK genes were not included in this figure. The evolutionary trajectory of WAK family in *A. thaliana* (At), *G. arboreum* (Ga), *G. raimondii* (Gr), and *G. hirsutum* (Gh). Numbers in circles represent the number of family members in each genome or subgenome, and the gene number for the most recent common ancestor of two diploid cotton species is shown in rectangles. Numbers on the line connecting each node with plus (+) and minus (−) signs indicate the numbers of duplicated and deleted genes, respectively.
The chromosomal distribution of *AtWAK* genes indicates a tandemly arrayed gene (TAG) cluster on chromosome 1 (He et al., 1999). We investigated the physical positions of *GhWAK* genes in the *G. hirsutum* genome and found that *GhWAK* distribute unevenly among six chromosomes with many members as TAG clusters (Figure 1C). *GhWAK1D, GhWAK2D, GhWAK3D*, and *GhWAK4D* present as a cluster of TAGs on the D10 chromosome. Notably, *GhWAK1D, GhWAK2D, GhWAK3D*, and *GhWAK4D* are clustered together on the phylogenetic tree with 83% to 93% identities between each other, suggesting these four paralogous *GhWAKs* likely were derived from one ancestor. Surprisingly, there are no corresponding homoeologous genes of these four *GhWAKs* on the A subgenome (Figure 1C). However, there are seven *GaWAKs* as a cluster of TAGs on chromosome 10 of *G. arboreum* (A-genome ancestor; Supplemental Figure 5A), suggesting that this cluster was lost during speciation of tetraploid *G. hirsutum*.

The other two separate clusters are composed of *GhWAK6D, GhWAK8D*, and *GhWAK10D* on the D02 chromosome and *GhWAK7A, GhWAK8A*, and *GhWAK10A* on the A02 chromosome. *GhWAK8A-GhWAK8D* and *GhWAK10A-GhWAK10D* are likely two pairs of positional homeologs with 89.46% and 94.33% protein identities, respectively (Figure 1C). *GhWAK6D* and *GhWAK7A* are also possible homoeologous genes with relatively high divergence (63.11% protein identity). Thus, they were given different names.

Interestingly, the closest orthologs of *GhWAK1D, GhWAK2D, GhWAK3D*, and *GhWAK4D* in *G. raimondii* (D-genome ancestor) are *Gorai.011G218500* and *Gorai.011G218100*, two TAGs on chromosome 11 (Supplemental Figure 5B), in accordance with the observation that the D-subgenome chromosome 10 of *G. hirsutum* has a high collinear relationship with the D-genome chromosome 11 of *G. hirsutum* (Figure 1C). *GhWAK7A* and *GhWAK8A* are *G. raimondii* homologs with 94.33% and 94.33% protein identities, respectively (Figure 1C). *GhWAK6D* and *GhWAK7A* are also possible homoeologous genes with relatively high divergence (63.11% protein identity). Thus, they were given different names.

WAK family proteins have been recognized to play an important role in disease resistance against fungal pathogens by several independent studies (Zuo et al., 2015; Shi et al., 2016; Hu et al., 2017; Saintenac et al., 2018; Yang et al., 2019). To unravel the involvement of *GhWAKs* in response to fungal pathogen infection, the transcriptional changes of *GhWAK* genes upon fungal pathogen infection were investigated. We first analyzed the published RNA-seq data sets from the cotton *G. hirsutum* ‘YZ-1’, which has been widely used for cotton transformation and regeneration, infected with the Vr991 isolate prevalently present in China (Zhang et al., 2018). This analysis revealed that eight out of 13 *GhWAKs* were upregulated at 6-, 12-, or 24-h post-inoculation (hpi), with *GhWAK7A* showing 12-fold induction at 6 hpi (Figure 2A).

We then confirmed the transcriptional changes of *GhWAK* genes using reverse transcription-quantitative PCR (RT-qPCR) with gene-specific primers in *G. hirsutum* ‘CA4002’, a U.S. cultivar, infected by the Vd ‘King’ isolate, a prevalent field isolate in Texas, USA. In addition to *GhWAK7A, GhWAK5A—* which shares a relatively high protein identity (61.16%) with *GhWAK7A*, but displayed no induction within 24 hpi—was selected for comparison. Consistent with the RNA-seq result, *GhWAK7A* showed enhanced expression at 1-d post-inoculation (dpi), whereas *GhWAK5A* was only induced at 7 dpi (Figure 2B).

The transcriptional changes of *GhWAK5A* and *GhWAK7A* in response to Vd infections prompted us to examine their functional involvement in cotton defense against fungal diseases. We silenced the individual members of *GhWAK5A* and *GhWAK7A* by VIGS. Three weeks after Agrobacterium infiltration, RT-PCR analysis confirmed the reduced transcripts of *GhWAK* genes in the true leaves of cognate VIGS plants compared with the control vector-inoculated plants (VIGS-Ctrl; Figure 2C). In addition, RT-qPCR was performed to examine the transcript levels of individual *GhWAK* homologs in roots, where vascular pathogens first infect the plant. The results showed that the transcripts of *GhWAK7A* and *GhWAK5A*, but not any of the other eight *GhWAK* homologs, were reduced in the cognate VIGS plants compared with the control plants (Figure 2D), confirming the efficiency and specificity of VIGS silencing in cotton roots.

Three weeks after VIGS, the plants were inoculated with spores of the Vd ‘King’ isolate by a root-dipping method (Gao et al., 2013a). Compared with control, *GhWAK7A*-silenced plants displayed more wilting and yellow leaves, and deeper vascular discoloration in stem tissues (Figure 2E). We quantified the disease severity with disease index (DI) via five scales of foliar
Figure 2. GhWAK7A Is Involved in Cotton Defense Responses against Fungal Wilt Diseases.

(A) Heat map of GhWAK family genes in RNA-seq data derived from cotton roots infected with the Vd V991 isolate (Zhang et al., 2018). The map was generated using the program Morpheus (https://software.broadinstitute.org/morpheus). Red color indicates relatively high expression and blue indicates relatively low expression. The corresponding value of fragments per kilobase of transcript per million is shown in the colored box.

(B) The expression patterns of GhWAK5A and GhWAK7A upon Vd infection. Two-week-old cotton seedlings were inoculated with the Vd ‘King’ isolate using a root-dipping method. The root samples were harvested at the indicated time-points for RT-qPCR. GhUBQ1 was used as an internal control. The data are shown as mean ± se from three independent repeats. Asterisks indicate a significant difference using one-way ANOVA (*P < 0.05; **P < 0.01).

(C) Expression of GhWAK genes in control and silenced plants measured by RT-PCR. Cotyledons from 2-week-old cotton were syringe-infiltrated with Agrobacterium carrying a VIGS construct to silence GhWAK7A (VIGS-GhWAK7A), GhWAK5A (VIGS-GhWAK5A), or a GFP vector control (Ctrl). Three weeks after VIGS, the second true leaves were harvested for RNA isolation. GhActin9 was used as an internal control. Data are shown as mean ± se from three independent repeats. Asterisks indicate significant differences from Ctrl using a two-tailed Student’s t test (*P < 0.05; **P < 0.01).

(D) VIGS efficiency and specificity of GhWAK7A and GhWAK5A in cotton roots by RT-qPCR analysis. Three weeks after VIGS infiltration, cotton roots were harvested for RNA isolation and RT-qPCR analysis. GhUBQ1 was used as an internal control. Data are shown as mean ± se from three independent repeats. Asterisks indicate significant differences from Ctrl using a two-tailed Student’s t test (*P < 0.05; **P < 0.01).

(E) Disease symptoms of leaves and stems in Vd-infected VIGS-GhWAK genes and control plants. Three weeks after VIGS, plants were inoculated with Vd ‘King’ isolate at an inoculum of $1 \times 10^6$ spores/mL using the root-dipping method. Images depicting the severity of plant wilting were taken at 14 dpi (left representation). Close-up views from the left representations are shown in the middle. Scale bar = 2 cm for plants. Vascular discoloration was observed in the stem 1.5 cm above the soil-line at 16 dpi under an optical microscope (right representation). Scale bar = 0.5 mm for stems.

(F) Disease symptoms of leaves and stems in Fov-infected VIGS-GhWAK genes and control plants. Three weeks after VIGS, plants were inoculated with Fov ‘CA10’ at $1 \times 10^7$ spores/mL using the root-dipping method. Images depicting the severity of foliar and plant wilting symptoms were taken at 20 dpi (left representation). Close-up views from the left representations are shown in the middle. Scale bar = 2 cm for plants. Vascular discoloration was observed in the stem, 1.5 cm above the soil-line, at 22 dpi (right representation). Scale bar = 0.5 mm for stems.

The above experiments were repeated at least three times with similar results.
symptoms (Xu et al., 2012). Quantification of DI further supported this conclusion (Figure 2F).

We next tested whether GhWAK7A and GhWAK5A contribute to cotton defense against another fungal wilt pathogen, Fov, which causes Fusarium wilt. Interestingly, VIGS-GhWAK7A plants became more susceptible to the Fov CA10 isolate with more wilting leaves and plants and darker vascular discoloration in stem tissues compared with control vector-inoculated plants (Figure 2G), whereas VIGS-GhWAK5A plants were more resistant to the Fov CA10 infection with fewer wilting leaves and less vascular discoloration compared with control plants (Figure 2G). The DI also indicated that VIGS-GhWAK7A plants were more susceptible, while VIGS-GhWAK5A plants were more resistant to the Fov CA10 infection than the control plants (Figure 2H).

Several WAKs and WAKLs have been reported to link to plant responses against abiotic stresses (Hou et al., 2005; Xia et al., 2018; Wang et al., 2019). However, VIGS-GhWAK7A plants showed comparable responses to VIGS-Ctrl plants during water-deprivation and 200 mM of NaCl salt treatment (Supplemental Figure 8). Taken together, the data indicate that GhWAK7A may not be involved in cotton tolerance to drought or salt stresses but plays a positive role in cotton resistance to both Vd and Fov, implying a potentially conserved mechanism in cotton responses to different fungal pathogens.

GhWAK7A Contributes to Chitin-Induced Immune Responses in Cotton

Chitin, a conserved fungal cell wall component, triggers immune responses in plants (Shinya et al., 2015; Kawasaki et al., 2017). The contribution of GhWAK7A to defense against two different fungal pathogens prompted us to test the involvement of GhWAK7A in chitin-induced immune responses. RT-qPCR analysis indicated that GhWAK7A, but not GhWAK5A, was induced as early as 0.5 h upon chitin treatment (Figure 3A).

The activation of mitogen-activated protein kinases (MAPKs) is one of the early immune responses triggered by the perception of chitin and other MAMPs (Yu et al., 2017). Chitin treatment induced rapid and transient activation of GhMPK3 and GhMPK6 in cotton detected by immunoblots using an anti-phosphorylated extracellular-regulated protein kinase 1/2 antibody (Supplemental Figure 9A). The activation of GhMPK3 and GhMPK6 was confirmed using VIGS-GhMPK3 or VIGS-GhMPK6 plants in which the protein levels of GhMPK3 or GhMPK6 were reduced (Supplemental Figures 9A and 9B). Compared with control, the silencing of GhWAK7A, but not GhWAK5A, resulted in compromised GhMPK6 and GhMPK3 activation without affecting the GhMPK6 or GhMPK3 protein levels (Figures 3B and 3C).

The production of ROS is another well-characterized immune response triggered by chitin and other MAMPs (Yu et al., 2017). The chitin-induced ROS production was lower in GhWAK7A-silenced cotton plants than that in the control plants (Figure 3D). Chitin treatment induced expression of GhWRKY30 and GhMPK3 in cotton, and the expression levels of both GhWRKY30 and GhMPK3 after chitin treatment were reduced in GhWAK7A-silenced cotton plants relative to control plants (Figure 3D). Collectively, these data indicate that GhWAK7A is involved in the chitin-induced immune responses.

Earlier studies suggested that AtWAKs bind pectin in plant cell walls and are involved in cell expansion during development and the responses to pathogens and wounding (Brutus et al., 2010; Kohorn, 2016; Riese et al., 2018). To examine whether the sensing of cell-wall-derived pectin may account for the role of GhWAK7A and GhWAK5A in defense responses, we examined MAPK activation triggered by OGs, pectin fragments derived from the partial hydrolysis of plant cell wall pectin (Galletti et al., 2009). OGs were generated by the digestion of polygalacturonic acid (Na+ salt) with Aspergillus niger endopolygalacturonase. The RT-qPCR data showed that the induction of AtFRK1 by OGs was similar to that by fig22 in Arabidopsis (Supplemental Figure 10A). High-performance anion-exchange chromatography with pulsed amperometric detection indicated OG oligomers with a distribution of the degree of polymerization between 6 and 20 and an enrichment in the degree of polarization around 12 (Supplemental Figure 10B). This is consistent with the analysis using electrospray ionization mass spectrometry (Supplemental Figure 10C). Cotton leaves treated with OGs triggered rapid GhMPK6 and GhMPK3 activation with a peak at 15 min (Supplemental Figure 10D), and ROS production (Supplemental Figure 10E) and transcriptional up-regulation of GhWRKY30 and GhMPK3 at 30 min in cotton (Supplemental Figure 10F).

However, compared with control plants, the silencing of GhWAK7A did not affect OG-induced MAPK activation (Figure 3F), ROS production (Figure 3G), and GhWRKY30 and GhMPK3 upregulation (Figure 3H). In addition, the fungal cell wall extracts from Fov ‘CA10’ (FovCWE) triggered immune responses, including MAPK activation. However, compared with control plants, the silencing of GhWAK7A or GhWAK5A did not affect FovCWE-induced MAPK activation (Figure 3I). This contrasts with the requirement of GhWAK7A in chitin-induced immune responses, suggesting that the function of GhWAK7A in plant defense might not be due to its role in response to cell-wall-derived components.

Identification and Functional Analysis of Chitin-Sensing Receptor Complex in Cotton

The Arabidopsis LYKs with the extracellular LysM motif compose a family of proteins with five members (Tanaka et al., 2013). AtCERK1 (AtLYK1), AtLYK4, and AtLYK5 were reported to function in chitin perception and signaling activation (Miya et al., 2007; Liu et al., 2012; Wan et al., 2012; Cao et al., 2014). Chitin-induced MAPK activation and ROS production were abolished in the atlyk1 mutant and atlyk4/5 double mutant and were reduced in the atlyk4 mutant compared with wild-type Columbia-0 (Col-0) plants (Supplemental Figures 11A and 11B; Cao et al., 2014; Xue et al., 2019). However, the atlyk5 (atlyk5-2) mutant exhibited a slight reduction in chitin-induced ROS production and no observable difference in chitin-induced MAPK activation compared with wild-type plants (Supplemental Figure 11). AtLYK4 and AtLYK5 function as the chitin receptor and AtCERK1 is likely a coreceptor or signaling partner of AtLYK4/AtLYK5 (Liu et al., 2012; Cao et al., 2014; Xue et al., 2019). We further tested whether the AtLYK4/5-AtCERK1 chitin sensory complex is involved in Arabidopsis response to Verticillium and Fusarium wilt pathogens. It has been reported that Vd could infect Arabidopsis (Veronese et al., 2003), and F. oxysporum (Fo) ‘5176’ is a pathogenic fungus on
Arabidopsis (Thatcher et al., 2012). The *atcerk1* and *atlyk4/5* mutants were more susceptible to *Vd* and *Fo* ‘5176' infections in terms of disease symptom development (Figure 4A) and DI (Figure 4B) compared to wild-type plants. Thus, Arabidopsis AtCERK1 and AtLYK4/5 play a positive role in resistance against *Verticillium* and *Fusarium* fungal pathogens.

**Figure 3.** The Involvement of GhWAK7A in the Chitin-Triggered but Not OG-Triggered Immune Responses in Cotton.

(A) The expression pattern of GhWAK5A and GhWAK7A upon chitin treatment. The leaf discs from cotton true leaves were soaked in distilled water overnight to eliminate wounding stress and treated with 100 μg/mL of chitin solution. Samples were harvested at the indicated time-points for RT-qPCR analysis. The data are shown as mean ± se from three independent repeats. Asterisks indicate a significant difference using a two-tailed Student’s t test (**) P < 0.01.

(B) Silencing GhWAK7A in cotton reduces chitin-induced MAPK activation. Cotton seedlings were silenced with GhWAK7A, GhWAK5A, or Ctrl by VIGS. The leaf discs from the second pair of true leaves were treated with 100 μg/mL of chitin at the indicated time-points. Phosphorylated GhMPK3 and GhMPK6 were detected by immunoblotting with an anti-phosphorylated extracellular-regulated protein kinase 1/2 (α-pERK1/2) antibody. The protein levels of GhMPK6 and GhMPK3 were detected with α-MPK6 and α-MPK3 antibodies, respectively. Protein loading is indicated by Ponceau S staining for Rubisco (RBC). The molecular mass (kDa) was labeled on the left of images (same for other immunoblots).

(C) The quantification of the relative intensities of immunoblot bands in (B). The values indicate the densitometry units of phosphorylated GhMPK3/6 (pGhMPK3/6) bands normalized to the protein inputs at 15 or 30 min after chitin stimulation. The data are shown as mean ± se from three independent repeats. Asterisks indicate a significant difference using one-way ANOVA (**P < 0.01).

(D) Silencing GhWAK7A in cotton dampens chitin-induced ROS production. Three weeks after VIGS, the leaf discs from the second true leaves were soaked in distilled water overnight to eliminate wounding stress and treated with 100 μg/mL of chitin. ROS production was measured at the indicated time-points. The data are shown as the mean ± se (n ≥ 8).

(E) Silencing GhWAK7A reduces chitin-induced GhWRKY30 and GhMPK3 expression. Three weeks after VIGS, true leaves were treated with 100 μg/mL of chitin and collected at 0 and 30 min for RT-qPCR analysis. GhUBQ1 was used as an internal control. The data are shown as mean ± se from three independent repeats. An asterisk indicates a significant difference using a two-tailed Student’s t test (*P < 0.05).

(F) Silencing of GhWAK7A or GhWAK5A by VIGS does not alter OG-induced MAPK activation in cotton leaves. Three weeks after VIGS, leaf discs from the second true leaves were treated with 100 μg/mL of OGs at the indicated time-points. MAPK activation assays were done as in (B).

(G) Silencing GhWAK7A in cotton does not affect OG-induced ROS production. After VIGS infiltration, the cotton leaf discs were treated with 100 μg/mL of OGs, and ROS production was measured as described in (D). The data are shown as the mean ± se (n ≥ 8).

(H) Silencing GhWAK7A in cotton does not affect OG-induced GhWRKY30 and GhMPK3 expression. Three weeks after VIGS, the cotton leaf discs were treated with 100 μg/mL of OGs and RT-qPCR analysis was performed as in (E). The data are shown as mean ± se from three independent repeats.

(I) Neither silencing GhWAK7A nor GhWAK5A alters FovCWE-induced MAPK activation in cotton. Three weeks after VIGS, the cotton leaf discs were treated with 1 mg/mL of FovCWE isolated from Fov ‘CA10'. The MAPK activation was detected as in (B).

The above experiments were repeated at least three times with similar results.
To uncover the chitin perception and signaling mechanism in cotton, we first performed an in silico analysis to identify the LYK family members in cotton using a similar method as we did for cotton WAKs. The Arabidopsis five LYK full-length amino acid sequences were used as BLASTp queries against three cotton databases in CottonFGD (https://cottonfgd.org/) with a threshold E-value < 1e-50. The candidates were further manually screened to retain only those proteins that contain a predicted signal peptide, LysM motif (or the extracellular CERK1 structure; Protein Data Bank [PDB] ID is PDB:4EBZ), transmembrane domain, and Ser/Thr kinase domain. Seventeen GhLYKs from the G. hirsutum genome were identified (Supplemental Table 7). Eight GaLYKs and 10 GrLYKs were identified from the genomes of G. arboreum and G. raimondii, respectively (Supplemental Tables 8 and 9).

To understand the evolutionary history of LYK gene family members, the 35 cotton LYKs and five Arabidopsis LYKs were subjected to a phylogenetic analysis using the program MEGA X. Interestingly, cotton LYKs can be classified into five clades with each of five Arabidopsis LYK proteins in one clade (Figure 4C, Supplemental Figure 12; Supplemental Table 10). This is different...
from the phylogenetic relationship of WAKs in cotton and Arabidopsis, in which none of the Arabidopsis WAKs interleave with cotton WAKs (Supplemental Figure 2). This indicates that LYK members may have evolved before cotton and Arabidopsis speciation, whereas WAKs may have evolved independently after cotton and Arabidopsis speciation.

GhLYKs were named considering the orthology to AtLYKs and homoeology between A- and D-subgenomes (Figure 4C; Supplemental Table 11). The number (1 to 5) is to indicate the clade relatedness to the five AtLYKs (I to V). Lowercase letters (a to c) are added after the number to differentiate genes in the same clade. An uppercase “A” or “D” is followed to indicate from either the A- or D-subgenome. In G. hirsutum, four proteins, including GhCERK1aA, GhCERK1aD, GhCERK1bA, and GhCERK1bD, are putative AtCERK1 orthologs with 50% to 60% protein identity (Figure 4C; Supplemental Table 12). Five GhLYK3, including GhLYK3aA, GhLYK3aD, GhLYK3bA, GhLYK3bD, and GhLYK3cD, are putative orthologs of AtLYK3; and two GhLYK2—GhLYK2A and GhLYK2D—could be inferred as AtLYK2 orthologs. Four proteins with 30% to 50% identity to AtLYK4, including GhLYK4aA, GhLYK4bA, GhLYK4cA, and GhLYK4cD, and two proteins with 56% identity to AtLYK5, including GhLYK5A and GhLYK5D, are clustered (Figure 4C; Supplemental Table 12). This is consistent with the analysis in Arabidopsis that AtLYK4 and AtLYK5 have a closer evolutionary relationship than the other three AtLYKs (Cao et al., 2014). The four GhCERK1 homoeologs showed an extracellular PDB:4EBZ structure (Figure 4C), which consists of three LysM motifs (Liu et al., 2012). An alignment of the GhLYK LysM motif sequences reveals four highly conserved residues (Y_D_N_)_ in the LysM domain. The phylogenetic analyses between GhLYK and AtLYK (Figure 4C) shows that the GhLYKs are more closely related, whereas LYK2, LYK4, and LYK5 are quite related in both Arabidopsis and cotton (Figure 4C).

The physical positions of GhLYKs distribute among eight chromosome pairs with nine genes in the A-subgenome and eight in the D-subgenome (Supplemental Figure 14). Unlike GhWAKs in which most of the genes are TAGs, only GhLYK4aA and GhLYK4bA are tandemly arrayed among all GhLYKs (Supplemental Figure 14). The phylogenetic analyses between GhLYKs from the A-subgenome and GaLYKs, and between GhLYKs from the D-subgenome and GrLYKs, suggest that seven out of nine GhLYK A-subgenome genes have the corresponding orthologs in G. arboreum, and all eight GhLYK D-subgenome genes have the corresponding orthologs in G. raimondii (Supplemental Figures 15A and 15B, and 16). The analyses revealed the trajectory of LYK gene number changes during the cotton genome evolution (Figure 4D). In addition, one gene (Ga05G2087.1) from G. arboreum, and two genes (Gora.0111G012100.1 and Gora.0062G245800.1) from G. raimondii, were lost during G. hirsutum speciation (Supplemental Figures 15A and 15B, and 16).

There are six orthologous LYK genes between G. arboreum and G. raimondii (Supplemental Figure 15C), which were considered as ancestor LYK genes in cotton (Figure 4D). Additionally, GhCERK1aA and GhLYK4bA are unique in G. hirsutum (Supplemental Figure 15A). GhLYK4bA clusters with GhLYK4aA on chromosome A11 and they share a high degree of sequence identity (85.9%; Supplemental Figure 14; Supplemental Table 12), suggesting that GhLYK4bA may have emerged via a local duplication event from GhLYK4aA, which is derived from Ga11G3609.1. Thus, LYKs and WAKs represent two unique family genes for studying genome evolution, gene birth, death, and divergence.

Silencing GhCERK1 or GhLYK5 Enhances Cotton Susceptibility to Fungal Wilt Diseases

The evolutionary conservation of cotton LYKs and Arabidopsis LYKs suggests that GhLYKs may exhibit a function that is similar to that found in their AtLYK orthologs. To investigate the potential contribution of GhLYKs to cotton fungal diseases, we analyzed the expression pattern of GhLYK genes in the RNA-seq data of cotton upon infection with the Vd V991 isolate (Zhang et al., 2018). All the GhLYK genes, except GhLYK3aD and GhLYK3aA, were up-regulated to different extents upon Vd infection at different time-points (Figure 5A). We further deployed VIGS to silence GhLYK1 (GhCERK1 hereafter), GhLYK2, GhLYK3, GhLYK4, and GhLYK5. Given that there are several copies of each GhLYK in the cotton genome, we selected conserved regions of the given gene for VIGS and attempted to silence all the copies of the corresponding genes; for example, VIGS-GhCERK1 would silence GhCERK1aA, GhCERK1aD, GhCERK1bA, and GhCERK1bD. Three weeks after Agrobacterium-mediated VIGS infiltration, RT-PCR analysis was performed to confirm the silencing efficiency (Figure 5B).

Compared with control plants, VIGS-GhCERK1 plants showed markedly more severe disease symptoms that included leaf chlorosis and wilting, stem vascular discoloration (Figure 5C), and an elevated DI after Vd ‘King’ isolate infection (Figure 5D). Similar results were consistently obtained in all seven biological repeats (Supplemental Table 13). We also observed that VIGS-GhLYK2 plants were more susceptible than control plants in five out of seven repeats, and VIGS-GhLYK5 plants were much susceptible in four out of seven repeats relative to control plants (Supplemental Table 13). Similar to Vd, Fov CA10 infection caused more severe infection in VIGS-GhCERK1 or VIGS-GhLYK5 plants compared with control plants as shown with more withering leaves and plants, darker stem discoloration, and elevated DI (Figures 5E and 5F). Among six repeats with Fov infections, VIGS-GhLYK5 plants showed susceptibility in five repeats and VIGS-GhCERK1 plants were susceptible in all repeats in terms of disease symptoms and indexes (Supplemental Table 14).

GhCERK1 and GhLYK5 Are Essential for Chitin-Triggered Immune Responses in Cotton

Given the result that GhCERK1 and GhLYK5 positively contribute to cotton defense against fungal wilt diseases, we further explored whether plants silenced for these genes showed altered responses to chitin treatment. Silencing of GhCERK1 (but not GhLYK2, GhLYK3, or GhLYK4) compromised chitin-induced MAPK activation compared with control plants (Figures 6A and 6B). In addition, silencing of GhLYK5 resulted in a slight, but reproducible, reduction of chitin-induced MAPK activation (Figures 6A and 6B).
Because AtLYK5 has an overlapping function with AtLYK4 in chitin perception and signaling (Cao et al., 2014), we designed a VIGS construct to silence both GhLYK4 and GhLYK5 simultaneously (VIGS-GhLYK4/5) and the VIGS efficiency was confirmed by RT-PCR (Supplemental Figure 17A). A significant reduction of chitin-induced MAPK activation was detected in VIGS-GhLYK4/5 plants, compared with VIGS-GhLYK4 or -GhLYK5, suggesting that cotton GhLYK4 and GhLYK5 exert redundant functions in chitin perception and signaling (Figures 6A and 6B).

Consistent with these observations, chitin-induced ROS production was largely compromised in the VIGS-GhCERK1 or VIGS-GhLYK4/5 plants and partially reduced in the VIGS-GhLYK5 plants (Figure 6C). Chitin-induced marker gene expression exhibited a similar trend in these GhLYK-silenced cotton plants. Compared

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**Figure 5.** Silencing GhCERK1 or GhLYK5 Increases Cotton Susceptibility to Fungal Wilt Pathogens.

(A) Heat map of GhLYK family genes in RNA-seq data derived from cotton roots infected with the Vd V991 isolate (Zhang et al., 2018). The map was generated from the program Morpheus (https://software.broadinstitute.org/morpheus). Red color indicates the relatively high gene expression level, and blue indicates a low expression. The corresponding fragments per kilobase of transcript per million value are shown in the colored box.

(B) The expression of genes in control and silenced plants upon VIGS. Cotyledons from two-week-old cotton plants were syringe-infiltrated with Agrobacterium carrying a VIGS construct to silence GhCERK1 (VIGS-GhCERK1), GhLYK2 (VIGS-GhLYK2), GhLYK3 (VIGS-GhLYK3), GhLYK4 (VIGS-GhLYK4), GhLYK5 (VIGS-GhLYK5), or a GFP control (Ctrl). The second true leaves were harvested at 3 weeks after VIGS infiltration for RNA isolation for RT-PCR analysis. GhActin9 was used as an internal control.

(C) Cotton leaf wilting and stem vascular discoloration symptoms upon Vd infection. Three weeks after VIGS, plants were inoculated with the Vd 'King' isolate at an inoculum of $1 \times 10^6$ spores/mL using a root-dipping method. Images depicting the severity of foliar wilt symptoms were taken at 13 dpi (left). Close-up views from the left are shown in the middle. Scale bar = 2 cm for plant images. Vascular discoloration was observed in the stem, 1.5 cm above the soil-line, at 19 dpi (right). Scale bar = 0.5 mm for stem images.

(D) Quantitative measurements of disease severity after Vd infection. The data are shown as the mean DI ± se from three independent repeats. An asterisk indicates a significant difference using one-way ANOVA (*P < 0.05).

(E) Cotton plant wilting and stem vascular discoloration upon Fov infection. After VIGS, plants were inoculated with Fov 'CA10' at $1 \times 10^7$ spores/mL using the root-dipping method. Images depicting the severity of plant wilt symptoms were taken at 20 dpi (left). Close-up views from the left are shown in the middle. Scale bar = 2 cm for plant images. Vascular discoloration was observed in the stem, 1.5 cm above the soil line at 27 dpi (right). Scale bar = 0.5 mm for stem images.

(F) Quantitative measurements of disease severity after Fov infection. The data are shown as mean DI ± se from three independent repeats. An asterisk indicates a significant difference using one-way ANOVA (*P < 0.05).

The above experiments (B) to (F) were repeated at least three times with similar results.
with control plants, the chitin-induced expression of GhWRKY30 and GhMPK3 was significantly compromised in GhCERK1- or GhLYK4/5-silenced plants (Figure 6D). Expression of GhLYK5 in atlyk4/5 mutant protoplasts restored chitin-induced MAPK activation (Figure 6E), implying a conserved function of GhLYK5 and AtLYK4/5 mediating the chitin-induced MAPK activation.
Chitin Is Perceived by the GhCERK1 and GhLYK5 Complex in Cotton

Arabidopsis AtLYK5-AtCERK1 is a sensory complex for chitin perception (Cao et al., 2014). We examined whether GhCERK1 and GhLYK5 function similarly in chitin perception and signaling in cotton. GhCERK1 (GhCERK1bd), GhLYK5 (GhLYK5D), or GhWAK7A with a hemagglutinin (HA)-tag (GhCERK1-HA, GhLYK5-HA, or GhWAK7A-HA) was expressed in cotton protoplasts and chitin magnetic beads were used to pull down the chitin binding proteins from total proteins isolated from protoplasts. Chitin pulled down GhLYK5 and GhCERK1, rather than GhWAK7A (Figure 6F), suggesting that GhCERK1 and GhLYK5 bind to chitin in cotton. Although GhWAK7A is involved in chitin signaling, it may not bind to chitin directly. Chitin pulled down more GhLYK5 than GhCERK1 (Figure 6F, top), suggesting that GhLYK5 might have a higher affinity to chitin than GhCERK1, as was observed in Arabidopsis (Cao et al., 2014).

Perception of chitin induces the association of AtCERK1 and AtLYK5, and the phosphorylation of AtCERK1 and AtLYK5 in Arabidopsis (Cao et al., 2014; Erwig et al., 2017). Using a Phos-tag gel, we could detect a clear mobility shift of GhCERK1 and GhLYK5 after chitin treatment, indicating chitin-induced phosphorylation of GhCERK1 and GhLYK5 (Figures 6G and 6H). A co-immunoprecipitation (co-IP) assay using cotton protoplasts expressing GhLYK5-HA and GhCERK1-FLAG revealed that GhLYK5 associated with GhCERK1 upon chitin elicitation (Figure 6I). The chitin-induced GhCERK1 and GhLYK5 phosphorylation were evidenced as mobility shifts in a regular SDS-PAGE analysis (Figure 6I, bottom two panels).

GhWAK7A Complexes with GhCERK1 and GhLYK5

Given that GhWAK7A is involved in chitin-induced early responses and the important roles of GhCERK1 and GhLYK5 in cotton chitin signaling, we tested the potential relationship of GhWAK7A with GhCERK1 and GhLYK5. A co-IP assay in cotton protoplasts co-expressing GhCERK1-FLAG with GhWAK7A-HA indicated that GhWAK7A-HA coimmunoprecipitated with GhCERK1-FLAG before and after chitin elicitation (Figure 7A). The association of GhWAK7A-HA and GhLYK5-FLAG independent of chitin treatment was observed in a similar assay (Figure 7B) and confirmed using reciprocally switched epitope tags in co-IP assays with GhWAK5A and GhRLP2, a cotton LRR-RLP, as specificity controls (Supplemental Figure 17B). A split-luciferase assay, in which GhWAK7A and GhLYK5 were fused to the N-terminal and C-terminal half of the luciferase gene respectively, further demonstrated the association of GhWAK7A and GhLYK5 in vivo (Figure 7C).

Furthermore, fluorescence lifetime imaging (FLIM)-Förster resonance energy transfer (FRET) measurements revealed that GhLYK5-GFP proteins are in close proximity to GhWAK7A-mCherry, but not to AtBIR2-mCherry, when co-expressed in Arabidopsis protoplasts (Figures 7D and 7E). The FRET efficiency, calculated based on the GFP fluorescence lifetime of GhLYK5-GFP and GhWAK7A-mCherry (8.85% ± 2.30%), is similar to that of AtBAK1-GFP and AtBIR2-mCherry (9.68 ± 1.68%; Figures 7D and 7E).

To test whether GhWAK7A directly interacts with GhCERK1 or GhLYK5, we performed an in vitro pull-down assay. The glutathione s-transferase (GST)-fused cytosolic domain of GhLYK5 (GST-GhLYK5CD), immobilized on glutathione-sepharose beads as the bait, specifically pulled down the GhWAK7A cytosolic domain fused to maltose binding protein (MBP) with an HA epitope at the C terminus (MBP-GhWAK7A CD-HA; Figure 7F). Similarly, GST-GhCERK1 CD could pull down MBP-GhWAK7A CD-HA proteins (Figure 7G), suggesting a direct interaction of the cytosolic domain of GhWAK7A with the cytosolic domains of GhLYK5 and GhCERK1. These findings imply that although GhWAK7A does not directly bind to chitin, it may modulate the cotton chitin perception complex of GhCERK1 and GhLYK5.

Interestingly, GST-GhCERK1 CD could not pull down MBP-GhLYK5 CD, supporting the specificity of an in vitro pull-down assay (Figure 7G). The data also indicate that the cytosolic domain of GhCERK1 and GhLYK5 cannot interact with each other directly in the absence of chitin treatment. This is in line with the notion that chitin binds and induces the complex formation of AtCERK1 and AtLYK5 via their extracellular domains in Arabidopsis (Cao et al., 2014; Xue et al., 2019).

GhWAK7A Modulates Chitin-Induced GhCERK1 and GhLYK5 Association and Phosphorylates GhLYK5

We further investigated the potential role of GhWAK7A in complexing with GhCERK1 and GhLYK5. When co-expressed with GhWAK7A in cotton protoplasts, the chitin-induced association of GhCERK1 with GhLYK5 was further enhanced (Figure 8A), suggesting that GhWAK7A likely promotes chitin-induced GhCERK1-GhLYK5 complex formation. Because GhWAK7A is a functional kinase (see below), we generated the GhWAK7A kinase-inactive mutant (GhWAK7A K451M) by mutating the Lys residue at position 451 (K451), the predicted ATP binding site to Met (M).

Using this mutant, we performed a co-IP assay to examine the potential role of the GhWAK7A kinase activity in the chitin-induced formation of the GhCERK1-GhLYK5 complex. The co-expression of GhWAK7A K451M dampened the chitin-induced association of GhCERK1-GhLYK5 (Figure 8B). Thus, GhWAK7A modulates chitin sensory complex GhLYK5-GhCERK1 in a kinase-dependent manner. We did not observe any notable effects of GhWAK7A or GhWAK7A K451M on the chitin-induced mobility shift of GhCERK1 or GhLYK5 (Figures 8A and 8B).

We further examined the potential autophosphorylation and transphosphorylation among GhWAK7A, GhCERK1, and GhLYK5. Similar to their counterparts in Arabidopsis (Tanaka et al., 2013; Cao et al., 2014), GST-GhLYK5 CD had no detectable kinase activity, and GST-GhCERK1 CD had moderate kinase activity in an in vitro kinase assay (Figures 8C and 8D). In contrast, MBP-GhWAK7A CD exhibited strong autophosphorylation activity, whereas MBP-GhWAK7A K451M-CD showed undetectable phosphorylation activity (Figure 8C). Significantly, MBP-GhWAK7A CD phosphorylated GST-GhLYK5 CD (Figure 8C). In addition, different from the phosphorylation of AtLYK5 by AtCERK1 in Arabidopsis (Erwig et al., 2017), neither MBP-GhCERK1 CD nor GST-GhCERK1 CD phosphorylated GST-GhLYK5 CD (Figure 8D; Supplemental Figure 17C).
To test the in vivo phosphorylation of GhLYK5 by GhWAK7A, GhLYK5-FLAG was co-expressed with vector control, GhWAK7A-HA, or GhWAK7A<sup>K44M</sup>-HA in cotton protoplasts with or without chitin treatment, and the phosphorylated GhLYK5-FLAG proteins after immunoprecipitation with FLAG-agarose were detected by anti-phosphoSer/Thr antibody. The results

**Figure 7.** GhWAK7A Interacts with GhCERK1 and GhLYK5.

(A) GhWAK7A associates with GhCERK1 in cotton protoplasts. GhWAK7A-HA and GhCERK1-FLAG were co-expressed in protoplasts, followed by 100 μg/mL of chitin treatment for 10 min. Co-IP was performed with α-FLAG agarose (IP: α-FLAG), and the proteins were analyzed by immunoblotting with an α-HA (IB: α-HA) or α-FLAG (IB: α-FLAG) antibody (top two panels). The input controls are shown in the bottom two panels.

(B) GhWAK7A associates with GhLYK5 independent of chitin elicitation in cotton protoplasts. GhWAK7A-HA and GhLYK5-FLAG were co-expressed in protoplasts followed by 100 μg/mL of chitin treatment for 10 min. The co-IP was performed as in (A).

(C) GhWAK7A associates with GhLYK5 with a split-luciferase assay. GhLYK5-Cluc-FLAG or GFP-Cluc-FLAG was co-expressed with GhWAK7A-Nluc-HA in protoplasts, and luciferase (LUC) activity was detected 12 h after transfection. The data are shown as mean ± SEM (n = 8). Asterisks indicate a significant difference using a two-tailed Student’s t test (**P < 0.01). Protein expression is shown on the bottom with immunoblots.

(D) FLIM-FRET analysis of GhLYK5 and GhWAK7A interaction in Arabidopsis protoplasts. The indicated proteins were transiently expressed in Arabidopsis protoplasts for 12 h before imaging analysis. Localization of the GhLYK5-GFP/AtBAK1-GFP and GhWAK7A-mCherry/AtBIR2-mCherry is shown with the first (green) and second column (red), respectively. The lifetime (τ) distribution (third column) and FRET efficiency (fourth column) are presented as pseudo-color images according to the scale. Paired GhLYK5-GFP and AtBIR2-mCherry was used as a negative control, and paired AtBAK1-GFP and AtBIR2-mCherry as a positive control (Halter et al., 2014). Scale bar = 10 μm.

(E) Quantification of lifetimes of GFP signals in (E). The GFP mean fluorescence lifetime (τ) values, ranging from 2.2 to 2.6 ns, were statistically analyzed and are shown as mean ± SD. “n” denotes the number of independent protoplasts. Asterisks indicate statistically significant differences (**P < 0.01), according to a two-tailed Student’s t test.

(F) GhWAK7A interacts with GhLYK5 in vitro. HA-tagged MBP-GhWAK7ACD (MBP-GhWAK7ACD-HA) was incubated with glutathione beads coupled with GST or GST-GhLYK5CD. The beads were collected and washed for immunoblots with α-HA or α-GST antibodies (left and middle). The protein inputs were determined by Coomassie Brilliant Blue (CBB) staining (right).

(G) GhWAK7A interacts with GhCERK1 in vitro. HA-tagged MBP-fusion proteins (MBP-GhWAK7ACD-HA or MBP-GhLYK5CD-HA) were incubated with glutathione beads coupled with GST or GST-GhCERK1CD. The pull-down assay was performed as in (F). The above experiments were repeated at least three times with similar results.
indicate that GhWAK7A enhanced, whereas GhWAK7A K451M reduced, the phosphorylation of GhLYK5 before and after chitin treatment (Figure 8E). Taken together, the data support the idea that GhWAK7A interacts with and phosphorylates chitin receptor GhLYK5.

DISCUSSION

Cotton fungal vascular diseases, Verticillium and Fusarium wilts, are among the most devastating diseases affecting the yield and quality of cotton worldwide. Chitin, a typical MAMP from the fungal cell wall, triggers defense responses through the activation of PTI. It remains unknown whether there is a conserved chitin perception and signaling system in cotton. In this study, we revealed the chitin sensory complex consisting of GhLYK4/5-GhCERK1 in cotton and established its important role in cotton defense against Verticillium and Fusarium wilt pathogens. Besides, we identified GhWAK7A, a WAK, that is involved in cotton defense against Vd and Fov by modulating chitin signaling (Figure 9). Without infection, GhWAK7A interacts with GhCERK1 and GhLYK5. Upon fungal pathogen invasions, chitin fragments released from the fungal cell wall are recognized by cotton GhLYK5, which stimulates GhLYK5 association with GhCERK1. Phosphorylation or scaffolding of GhLYK5 by GhWAK7A might promote GhLYK5–GhCERK1 complex formation, which further activates the cytoplasmic signaling events, including ROS production, MAPK activation, and the expression of defense-related genes, to fend off Vd and Fov infections (Figure 9). Our study reveals that the role of
GhWAK7A in chitin signaling elucidates the potential mechanism of cotton responses to Vd and Fov, and provides genetic resources to improve cotton resistance to these two devastating fungal diseases.

Our genome-wide analysis indicates that both WAK and LYK family genes occurred as a major expansion in terms of gene number after cotton speciation from Arabidopsis, which is consistent with the notion that G. raimondii and G. arboreum underwent cotton-specific, whole-genome duplication ∼16.6 million years ago after the paleohexaploidization event common to all eudicots (Wang et al., 2012). The cotton LYK family was divided into five clades corresponding to the five members in Arabidopsis, indicating that the five LYK members had already evolved before cotton and Arabidopsis speciation. The high syntenic relationships between A-subgenome GhLYKs and GaLYKs, and between D-subgenome GhLYKs and GrLYKs, exemplified that the hybridization of an A-genome ancestor resembling G. arboreum with a D-genome ancestor resembling G. raimondii gave rise to the speciation of G. hirsutum (Paterson et al., 2012).

Different from LYKs, WAKs may have evolved independently after cotton and Arabidopsis speciation. Most cotton WAKs are clustered in a pattern that is consistent across the tetraploid and diploid species, similar to the WAK family distribution in Arabidopsis and rice (He et al., 1999; Zhang et al., 2005). Thus, tandem duplication plays a dominant role in the expansion of WAKs in different plant species, including both monocots and dicots. The different numbers of WAKs among different cotton species and their phylogenetic comparisons suggest that a large portion of WAKs in G. arboreum, especially those in chromosome 10, were lost during the polyploidization and speciation of G. hirsutum. This may contribute to the reduction of the G. hirsutum genome size after allopolyploidization (Li et al., 2015). Moreover, gene translocations were evident in several WAKs during the speciation of G. hirsutum. The conservation of LYKs and the divergence of WAKs in different plant species may support their biological functions, with LYKs as chitin receptors perceiving conserved fungal MAMPs and WAKs as the signaling components modulating PTI signaling.

LYKs, a type of RLK with extracellular LysM-domains, function in β-1,4-linked N-acetylglucosamine-containing microbial pattern perception, signaling, and plant immunity to fungal pathogens (Tanaka et al., 2013; Kawasaki et al., 2017). Most GhLYKs are upregulated upon Vd infection in cotton. GhCERK1 and GhLYK5 are required for cotton defense against fungal wilt pathogens. CERK1 is a critical component for chitin signaling in rice and Arabidopsis (Miya et al., 2007; Shimizu et al., 2010). GhCERK1, with 60% protein identity to AtCERK1, was demonstrated here to be essential for chitin-induced defense responses including MAPK activation, ROS production, and defense gene expression against fungal infection. AtLYK4 and AtLYK5 are involved in chitin sensing and share overlapping functions (Wan et al., 2012; Cao et al., 2014), and are required for plant defense against Vd and Fo ‘5176’. The simultaneous knockdown of GhLYK4 and GhLYK5 (GhLYK4/5) exhibited compromised chitin-induced responses in cotton, similar to the atlyk4/5 double mutant. AtLYK5 exhibited higher chitin binding affinity than AtCERK1, and forms a chitin-induced complex with AtCERK1 (Cao et al., 2014). Similarly, in cotton, GhLYK5 likely binds to chitin with higher affinity than GhCERK1 and associates with GhCERK1 in a chitin-dependent manner. Chitin induces the phosphorylation of GhLYK5 and GhCERK1 in vivo. In Arabidopsis, although the AtLYK5 kinase

Figure 9. A Proposed Model for GhWAK7A-Mediated Chitin Signaling in Cotton Defense against Fungal Pathogens.

(Left, without infections, GhWAK7A associates with GhLYK5 and GhCERK1, respectively. Right, upon fungal pathogen invasions, chitin fragments released from the fungal cell wall are recognized by cotton GhLYK5, which stimulates GhLYK5 phosphorylation by GhWAK7A and association with GhCERK1. The phosphorylated GhLYK5 might promote and/or scaffold GhLYK5-GhCERK1 complex formation, which further activates the cytoplasmic signaling events, including ROS production, MAPK activation, and the expression of defense-related genes, to fend off Vd and Fov infections.)
domain is inactive, it is required for chitin-induced association with AtCERK1, AtCERK1 phosphorylation, and activation of defense responses including ROS production and MAPK activation (Cao et al., 2014). Chitin-induced AtLYK5 phosphorylation is AtCERK1-dependent, which phosphorylates AtLYK5 in vitro (Erwig et al., 2017). Different from the observation in Arabidopsis that AtCERK1 phosphorylates AtLYK5 in vitro (Erwig et al., 2017), GhCERK1 has a moderate kinase activity, but it cannot phosphorylate GhLYK5. The data suggest that another kinase in the chitin sensory complex, likely GhWAK7A identified in our study, could phosphorylate and/or scaffold GhLYK5; this may promote chitin-induced GhLYK5-GhCERK1 complex formation, thereby activating downstream signaling.

WAKs compose a unique group of RLKs that likely associate with the pectin in the cell wall, and are necessary for both cell expansion during development and mediation of the response to pathogens (Kohorn and Kohorn, 2012). In cotton, eight GhWAKs were upregulated within 1 d after infection with Vd. Among them, GhWAK7A positively regulates cotton defense against fungal pathogens, Vd and Fov. Similarly, members of rice OsWAKs were transcriptionally upregulated by chitin treatment and quantitatively contribute to resistance against rice blast fungus Magnaporthe oryzae (Delteil et al., 2016). GhWAK7A is involved in chitin-induced immune responses in cotton but does not bind to chitin directly. Furthermore, GhWAK7A interacts with GhCERK1 and GhLYK5 and phosphorylates GhLYK5 in vitro. GhWAK7A promotes chitin-induced GhCERK1-GhLYK5 dimerization depending on the GhWAK7A kinase activity. The data suggest that GhWAK7A contributes to the chitin-induced phosphorylation of GhLYK5. This was further supported by the in vivo phosphorylation study in which GhWAK7A increased whereas the kinase-inactive GhWAK7A K405M reduced phosphorylation of GhLYK5. In light of the observations that the AtLYK5 kinase domain and AtCERK1 phosphorylation are required for the AtLYK5-AtCERK1 complex formation and initiating chitin signaling (Cao et al., 2014; Liu et al., 2018), it is tempting to speculate that the phosphorylation and/or scaffolding of GhLYK5 by GhWAK7A promotes GhLYK5 association with GhCERK1 upon chitin perception, which subsequently activates downstream signaling.

WAKs have been reported to be associated with the perception of OGs and cell wall components in Arabidopsis (Kohorn and Kohorn, 2012). We tested whether GhWAK7A is involved in the responses triggered by OGs, the potential ligand of AtWAK1 (Brutus et al., 2010). Silencing GhWAK7A did not affect OG-triggered MAPK activation, ROS production, or gene expression, suggesting that GhWAK7A might not be involved in OG responses. Besides, GhWAK7A did not contribute to the MAPK activation triggered by Fov, an unknown elicitor isolated from Fov cell wall. Alternatively, GhWAK7A might perceive an unknown ligand from Fov to activate defense signaling. Nevertheless, our study sheds light on the underlying mechanisms of WAKs in plant disease resistance against various fungal pathogens.

METHODS

Cotton WAK and LYK Family Gene Identification

The identification and phylogenetic analyses of cotton (Gossypium spp) WAK and LYK families were conducted among three cotton species—alloidiploid Gossypium hirsutum, diploid Gossypium arboreum, and Gossypium raimondii. The five Arabidopsis (Arabidopsis thaliana) WAK (WAK1-AT1G21250, WAK2-AT1G21270, WAK3-AT1G21240, WAK4-AT1G21210, and WAK5-AT1G21230) or LYK (CERK1-AT3G21630, LYK2-AT3G91840, LYK3-AT1G51940, LYK4-AT2G23770, and LYK5-AT2G33580) protein sequences were used as the queries for the BLASTp search against three cotton protein databases: G. hirsutum (AD; NAU assembly), G. arboreum (A; CRI assembly), and G. raimondii (D; JGI assembly) employing a threshold E-value < 1e-50 from the CottonFSG (https://cottonfgd.org/).

The candidates from all five queries for WAK or LYK were combined to form the primary list. From this list, protein sequences found to not belong to either the WAK or the LYK family, based on the annotation or the re-duplicated proteins, were deleted. Next, we manually checked each protein motifs using the programs SMART (http://smart.embl-heidelberg.de) and Interpro (https://www.ebi.ac.uk/interpro/) prediction. Proteins with a signal peptide, transmembrane domain, a kinase domain, and extracellular WAK, GUB, EGF, and EGF_Ca2⁺ domains for WAK, or LysM motif, or an annotated similarity to the PDB:4EBZ structure for LYK, were regarded as cotton WAK or LYK family proteins. Using this refined set of putative WAK and LYK full-length protein or kinase domain sequences, phylogenetic trees were built by the neighbor-joining method with 1,000 bootstrap replicates using the program MEGA X (Kumar et al., 2018). The exon–intron structures were analyzed by aligning the genomic DNA sequences with their corresponding coding sequences using the Gene Structure Display Server (v2.0; http://gsds.cbi.pku.edu.cn/).

Plant Materials and Growth Conditions

G. hirsutum ‘CA 4002’ (PI 665226) was grown in 3.5-inch square pots containing PRO-LINE C/25 soil (Jolly Gardener) at 23°C, 30% humidity, and 100 μM m⁻² s⁻¹ light (bulb type; Philips F40T12/DX) with a 12-h light/12-h dark photoperiod. Two-week-old cotton plants were used for Agrobacterium (Agrobacterium tumefaciens)-mediated VIGS assays and protoplast isolation. Three weeks after VIGS, plants were inoculated with Verticillium dahliae (Vd) ‘King’ isolate or Fusarium oxysporum f. sp. vavinctum (Fov) CA10.

Arabidopsis mutants atcerk1 (GABI-KAT 096F09), atlyk4 (CS5805683), atlyk5-2 (SALK_131911C), and atlyk4/5 (atlyk4/5) were obtained from Gary Stacey (Cao et al., 2014). The mutants and wild-type plants were grown in soil (Metro Mix 366) in a growth room at 23°C, 45% humidity, and 75 μE m⁻² s⁻¹ light with a 12-h light/12-h dark photoperiod. Three-week-old plants were inoculated with Vd ‘King’ isolate or Fusarium oxysporum (Fo) ‘5176’. Four-week-old plants were used for chitin-induced MAPK activation and ROS production assays.

Fungal Preparation and Inoculation

Vd ‘King’ isolate (Terry Wheeler, Texas A&M), Fov CA10 (Mike Davis, University of California at Davis), and Fo ‘5176’ (Lijun Ma, University of Massachusetts at Amherst) were grown on potato dextrose agar plates (Difco) for 4 d at room temperature (23°C) or 28°C for Fov and Fo ‘5176’. The hyphae of Vd were inoculated in Czapek medium (NaNO₃, 0.2% [w/v]; MgSO₄, 0.05% [w/v]; KH₂PO₄, 0.1% [w/v]; FeSO₄, 0.001% [w/v]; KCl, 0.05% [w/v]; Suc, 3% [w/v]; pH 7.3 ± 0.2) and incubated in a shaker (120 rpm) at 23°C for 5 d. The hyphae of Fov and Fo ‘5176’ were inoculated in potato dextrose broth (Difco) and incubated in a shaker (120 rpm) at 28°C for 5 d. The spores were collected from the medium by filtering through Miracloth (Millipore). After centrifugation at 4,000 rpm for 15 min, the spores were resuspended with sterile water and adjusted to the final concentration. The inoculum used for Vd inoculation was 1 × 10⁶ spores/mL and 1 × 10³ spores/mL for Fov and Fo ‘5176’.

The root-dipping method was used for both cotton and Arabidopsis inoculation as previously reported by Gao et al. (2013a, 2013b). Briefly,
three weeks after VIGS, cotton seedlings were removed from soil and the roots were dip-infected with Vd or Fov spore suspension for 3 min. The seedlings were then replanted in fresh soil. For Arabidopsis, 3-week-old seedlings were gently uprooted from soil and the roots were briefly washed with water to remove big chunks of soil. After the removal of excess water with paper towels, the roots were immersed in the Vd or Fov ‘5176’ spore suspension for 3 min and then planted in pots with pre-wet soil. The cotton and Arabidopsis seedlings inoculated with Vd were put back to the same growth room as they were before the infection, respectively. After inoculation with Fov or Fov ‘5176’, plants were transferred into a growth chamber at 28°C, 50% humidity, and 100 μE m⁻² s⁻¹ light with a 12-h light/12-h dark photoperiod.

Hydroponic Growth of Cotton and Salt Treatment

The hydroponic growth and salt treatment of cotton were performed as previously described by Mu et al. (2019) with minor modifications. G. hirsutum ‘CA 4002’ (PI 665226) seeds were surface-sterilized by gently shaking in 9% (v/v) H₂O₂ for 20 min, rinsed five times with sterile water, and covered with two-layer, pre-wet paper towels in a box at 28°C under 80% light. Three d post germination, the uniformly germinated seedlings were transplanted and suspended into containers with Hoagland solution (Micronutrients: 20 μM of H₃BO₃, 1 μM of ZnSO₄·7H₂O, 0.1 μM of CuSO₄·5H₂O, 1 μM of MnSO₄·H₂O, and 5 mM of (NH₄)₂MgO₄:; Macronutrients: 0.1 mM of EDTA FeNa, 2.5 mM of Ca(NO₃)₂·4H₂O, 0.5 mM of NH₄H₂PO₄, 1 mM of MgSO₄·7H₂O, and 2.5 mM of KNO₃). The Hoagland solution was agitated to ensure the proper root aeration and replaced freshly once a week. Seedlings were grown at 23°C, 60% humidity, and 80 μE m⁻² s⁻¹ light with a 14-h light/10-h dark photoperiod. Three d after transplantation, cotton cotyledons were infiltrated with Agrobacterium for infection. The disease severity of cotton plants was evaluated via DI based on the following formula: DI = (1n₁ + 2n₂ + 3n₃ + 4n₄) × 100/N₁, where n₁, n₂, n₃, and n₄ is the number of plants in the indicated scale, and N₁ is the total number of plants tested. For stem discoloration observation, the transaction slices of fresh stems ~1.5 cm above the soil-line were collected and examined under an optical microscope (model no. SZX10; Olympus).

The disease scales for Arabidopsis disease investigation are as follows: 0 = healthy plants; upon fungal infection, 1 = 0% to 25% of the leaves show chlorosis around the vein of leaves; 2 = 25% to 50% of the leaves show chlorosis or wilting; 3 = 50% to 75% of the leaves show chlorosis or wilting; and 4 = 75% to 100% of the leaves show chlorosis or wilting. The DI was calculated using the formula described above.

Elicitor Preparations

Chitin fragments were suspended in sterile water after sonication of chitin from shrimp shells (Sigma-Aldrich) and used at a final concentration of 100 μg/mL. OGs were obtained from pure polygalacturonate acid (Afas Aesar) by enzymatic digestion with purified polygalacturonase from Aspergillus niger (AnPGII), dialysis, and ethanol precipitation, as previously described by Pontiggia et al. (2015). High-performance anion-exchange chromatography with pulsed amperometric detection profiles and electrospray ionization mass spectrometry spectra of OGs were obtained in an electrospray ionization LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) in a positive ion mode as previously described by Benediti et al. (2017). OGs were dissolved in sterile water at a final concentration of 100 μg/mL, FovCWE from Fov ‘CA10’ was prepared according to Davies et al. (2006), and dissolved in sterile water at a final concentration of 1 mg/mL.

Plasmid Construction

The translated regions of the A- and D-subgenus copies and homoeologs of GhCERK1 (439 bp), GHLYK2 (472 bp), GHLYK3 (539 bp), GHLYK4 (281 bp), and GHLYK5 (636 bp), and the fragments of GhWAK5A (446 bp) and GhWAK7A (445 bp) were amplified from cotton (G. hirsutum) cDNA, and inserted into the pYL156 (pTRV-RNA2) vector with restriction-enzyme EcoRI and KpnI digestion for VIGS assays. The VIGS-GhCERK1, GhMPK6, and GhMPK3 constructs were previously reported by Gao et al. (2011) and Li et al. (2017a). To generate the VIGS-GHLYK4/GhLYK5 (GhLYK4/5) construct, fragments of GHLYK4 (336 bp) and GHLYK5 (279 bp) were PCR-amplified from cotton cDNA, digested with EcoRI and Ncol for the GHLYK5 fragment, and Ncol and KpnI for the GHLYK4 fragment, and ligated simultaneously with the pYL156 (pTRV-RNA2) vector-digested with EcoRI and KpnI. The full-length GHLYK1, GHLYK5, GHWAK7A, GHWAK5A, and GHRLP2 genes were amplified from cotton cDNA with primers containing BamHI, SpeI, and Ncol in the forward primers and EcoRV, Stul, or Smal in the reverse primers, and introduced into the plant gene expression vector pHBT with an HA, FLAG, or GFP epitope-tag at the C terminus. pHBT-p35S::GFP-Cluc-FLAG was generated by PCR-amplifying GFP from pHBT-p35S::GFP and sub-cloned into pHBT-p35S::Cluc-FLAG by BamHI and Spel digestion. Cluc (1,291 to 1,746 bp) was amplified from pHBT-pFRK1::LUC, pHBT-p35S::GHLYK5-Cluc-FLAG was generated by PCR-amplifying GHLYK5 from pHBT-p35S::GHLYK5-HA and sub-cloned into pHBT-p35S::Cluc-FLAG using a one-step cloning kit by BamHI and Spel digestion. Nluc (457 to 1,746 bp) was amplified from pHBT-pFRK1::LUC, pHBT-p35S::GhWAK7Nluc-HA was generated by PCR-amplifying GhWAK7A from pHBT-p35S::GhWAK7A-HA and sub-cloned into pHBT-p35S::Nluc-FLAG using a one-step cloning kit by BamHI and Spel digestion.

For FLIM-FRET constructs, genes of interest tagged by the fluorescence protein GFP or mCherry were developed according to Bajari et al. (2016). Briefly, AtBAK1 fragment was released from pHBT-p35S::AtBAK1-FLAG by BamHI and Stul digestion and ligated into the pHBT vector with a GFP epitope-tag at C terminus, resulting in pHBT-p35S::AtBAK1-GFP. The GFP-Nos terminator fragment was released from pHBT-p35S::AIBAK1-GFP by Stul and EcoRI digestion, and ligated into pHBT-p35S::GHLYK5-HA with the same enzyme digestion, resulting in pHBT-p35S::GHLYK5-GFP. Similarly, the AtBIR2 fragments were released from pHBT-p35S::AIBIR2-FLAG by BamHI and Stul digestion and ligated into the pHBT vector with a mCherry epitope-tag at C terminus, resulting in pHBT-p35S::AIBIR2-mCherry. The mCherry-Nos terminator fragment was released from pHBT-p35S::AIBIR2-mCherry by Stul and EcoRI digestion, and ligated into pHBT-p35S::GhWAK7A-HA with the same enzyme digestion, resulting in pHBT-p35S::GhWAK7A-mCherry. The point mutation of GhWAK7A(457-154) was generated by a site-directed mutagenesis kit using the wild-type GhWAK7A plasmid as the template. For Escherichia coli fusion protein constructs, the cytosolic domains of GhCERK1 (703 to 1,860 bp), GHLYK5 (922 to 2,010 bp), GHWAK7A (1,120 to 2,256 bp), and GHWAK7A(457-154) (1,120 to 2,256 bp) were amplified with primers containing BamHI or BglII in the forward primers and EcoRV or Stul in the reverse
primers, and introduced into a modified GST fusion protein expression vector GEX4T-1 (Pharmacia) or a modified pMAL-c2 vector (New England Biolabs) with BamHI and StuI digestion. The recombinant fusion protein expression vectors were introduced into E. coli strain BL21 (DE3). Expression of fusion proteins and affinity purification were performed following the standard protocol provided by the manufacturers. The primers for cloning and point mutations are given in Supplemental Table 15, and all the clones were confirmed by Sanger-sequencing.

RNA Isolation and RT-qPCR Analysis

To measure Vd-induced gene expression, 2-week-old cotton seedlings were inoculated with Vd ‘King’ isolate spores by the root-dipping method (Gao et al., 2013a). Root samples were harvested at the indicated time-points. To evaluate gene silence efficiency, three weeks after VIGS infiltration, the second true leaves or roots were collected. For chitin- or Ogs-induced gene expression in VIGS-silenced cotton, three weeks after Agrobacterium infiltration, 10 leaf discs (7.5-mm diameter) from the second true leaves of three individual plants for each sample were soaked in 100 μg/mL of chitin or 100 μg/mL of Ogs solution and harvested at 0 and 30 min. Cotton total RNAs were extracted from roots and leaves using a Spectrum Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer’s protocol and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNAs were then reverse-transcribed to synthesize the first-strand cDNAs with M-MuLV Reverse Transcriptase and oligo (dT) primers after RNase-free DNase I (New England Biolabs) treatment. RT-qPCR analysis was performed with the primers listed in Supplemental Table 15, using iTaq SYBR green Supermix (Bio-Rad) and a CFX384 Real-Time PCR System (Bio-Rad) following a standard protocol. The expression of each gene was normalized to the expression of GhUBQ1.

Agrobacterium tumefaciens-Mediated VIGS

Plasmids containing binary TRV vectors pTRV-RNA1 and pTRV-RNA2 derivatives, pYL156-GhCERK1, pYL156-GhLYK2, pYL156-GhLYK3, pYL156-GhLYK4, pYL156-GhLYK5, pYL156-GhWAK7A, pYL156-GhWAK7, pYL156-GhLYK5A, pYL156-GhLYK4/5, pYL156-GhCLA1, and pYL156-GFP (Ctrl) were introduced into Agrobacterium strain GV3101 by electroporation. The recombinant fusion protein expression vectors were introduced into Agrobacterium strain GV3101 by electroporation. The leaf discs (7.5-mm diameter) from the second true leaves of VIGS-silenced plants were subjected to elicitor treatments or inoculation with the inducer. The silenced of GhCLA1, which leads to a plant albino phenotype, was included as a visual marker for VIGS efficiency. Three weeks after VIGS, the silenced plants were subjected to elicitor treatments or inoculation with the indicated fungal pathogens.

MAPK Assay

The leaf discs (7.5-mm diameter) from the second true leaves of VIGS cotton or 4-week-old Arabidopsis plants were soaked in distilled water overnight, then treated with 100 μg/mL of chitin, 100 μg/mL of Ogs, or 1 mg/mL of FovCWE at the time-points indicated in each figure. Each sample contained two leaf discs from different plants and was ground in 100 μL of SDS buffer (62.5 mM of Tris-HCl at pH 6.8, 1% [w/v] SDS, 0.025% [w/v] bromophenol blue, 10% [v/v] glycerol, 4% [v/v] β-mercaptoethanol, 1× protease inhibitor, 2 μM of NaF, and 2 μM of Na3VO4) before being boiled at 95°C for 10 min. This boiled homogenate was centrifuged at 14,000g for 10 min at 4°C, and the supernatant was collected and loaded onto 10% SDS-PAGE gels before transfer to a polyvinylidene difluoride membrane, which was subsequently blotted using an anti-phosphorylated extracellular-regulated protein kinase 1/2 antibody (cat. no. 9101, dilution 1:2,000 [v/v]; Cell Signaling) for the detection of pMPK3/6, an α-MPK6 antibody (cat. no. A7104, dilution 1:10,000 [v/v]; Sigma-Aldrich) for the detection of MPK6 proteins, or an α-MPK3 antibody (cat. no. M8318, dilution 1:4,000 [v/v]; Sigma-Aldrich) for MPK3 proteins.

ROS Assay

ROS burst was determined by a luminol-based assay. The third and fourth pairs of true leaves from 4-week-old Arabidopsis or the second true leaves from cotton were excised into leaf discs (5-mm diameter). These discs were subjected to overnight incubation in a 96-well plate with 100 μL of distilled water to allow the immediate wounding effects to subside. Next, the water in each well was replaced by 100 μL of reaction solution containing 50 μM of luminol and 10 μg/mL of horseradish peroxidase (Sigma-Aldrich) supplemented with 100 μg/mL of chitin or 100 μg/mL of Ogs. The measurement was conducted immediately after adding the solution with a luminometer (model no. Victor X3; Perkin-Elmer), with a 1-min interval reading time for a period of 20 min for cotton or 30 min for Arabidopsis. The values for ROS production from each line were indicated as means of relative light units.

Chitin Binding Assays

The in vivo chitin binding assay was performed as described in Petutschnig et al. (2010) and Gu et al. (2017) with modifications. The GhCERK1, GhLYK5, and GhWAK7A in pHBT vector with an HA tag and vector control were expressed in 500 μL of cotton protoplasts (2×10^6 cells/mL) for 12 h. The cells were lysed by extraction buffer (100 mM of NaCl, 1 mM of EDTA, 20 mM of Tris-HCl at pH 7.5, 2 mM of NaF, 2 mM of Na3VO4, 1 mM of DTT, 0.5% [v/v] Triton X-100, 10% [v/v] glycerol, and 1× protease inhibitor). The cell lysis was centrifuged at 12,500g at 4°C for 15 min, the supernatant mixture was incubated with chitin magnetic beads (New England Biolabs) at 4°C for 3 h. The incubated beads were rinsed five times with washing buffer (100 mM of NaCl, 1 mM of EDTA, 20 mM of Tris-HCl, and 0.1% [v/v] Triton X-100). After 95°C boiling for 10 min in SDS loading buffer (62.5 mM of Tris-HCl pH 6.8, 1% [v/v] SDS, 0.025% [v/v] bromophenol blue, and 10% [v/v] glycerol), the immunoprecipitated and input proteins were analyzed by immunoblotting with an α-HA antibody (cat. no. 12013819001, dilution 1:2,000 [v/v]; Roche).

Co-IP Assay

Cotton protoplasts were isolated from cotyledons of 2-week-old seedlings (Gao et al., 2011), transfected with indicated constructs (1 mL of protoplasts with 2×10^6 cells), incubated for 12 h and followed by treatment with 100 μg/mL of chitin for 10 min. Samples were collected by centrifugation at 2,000×g at 4°C for 5 min, and 1 μL of resuspended protoplasts was mixed with 2 μL of 3× lysis buffer and then centrifuged. The supernatant was mixed with 3 μL of anti-HA antibody (cat. no. 12013819001, dilution 1:2,000 [v/v]; Roche), α-FLAG (cat. no. A8592, dilution 1:2,000 [v/v]; Sigma-Aldrich) or α-GFP (cat. no. 11814460001, dilution 1:2,000 [v/v]; Roche) antibody as indicated.

In Vitro Pull-Down Assay

The GST, GST-GhCERK1(1CO), and GST-GhLYK5(2CD) proteins were purified with Pierce glutathione agaroses (Thermo Fisher Scientific), and the MBP-GhLYK5(2CD) and MBP-GhWAK7A(2CD) proteins were purified with amylose
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resins (New England Biolabs) according to the protocols from the manufacturers. MBP-GhWAK7A and MBP-GhLYK5 were used as prey and pre-incubated with glutathione agaroses in 300 µL of co-IP buffer (20 mM of Tris-HCl at pH 7.5, 100 mM of NaCl, 1 mM of EDTA, 10% [v/v] glycerol, 0.5% [v/v] Triton X-100, 1 × protease inhibitor, 1 mM of DTT, 0.2% [v/v] of NaVO₃, and 2 mM of MgCl₂) for 0.5 h at 4°C. The supernatants after centrifugation were collected and incubated with 5 µL of pre-washed GST, GST-GhCERK1, or GST-GhLYK5 agarose beads for an additional 1 h.

The beads were collected and washed three times with washing buffer (200 mM of Tris-HCl at pH 7.5, 100 mM of NaCl, 1 mM of EDTA, 0.1% [v/v] Triton X-100), and once with 50 mM of Tris-HCl at pH 7.5. The MBP proteins were tagged with an HA epitope at the C terminus and were detected with an α-HA antibody (cat. no. 12013819001, dilution 1:2,000 [v/v]; Roche) by immunoblotting. GST fusion proteins are shown by immunoblotting with an α-GST antibody (cat. no. SC-53909, dilution 1:2,000 [v/v]; Santa Cruz Biotechnology).

Split-Luciferase Assay

GhWAK7A-Nuc-HA was co-expressed with GhLYK5-Cluc-LUC or GFP-CLuc-LUC in 200 µL of cotton protoplasts (2 × 10⁵ cells/mL) for 12 h. The cells were lysed in extraction buffer (100 mM of NaCl, 1 mM of EDTA, 20 mM of Tris-HCl at pH 7.5, 2 mM of NaF, 2 mM of Na₃VO₄, 1 mM of DTT, 0.5% [v/v] Triton X-100, 10% [v/v] glycerol, and 1 × protease inhibitor). The cell lysate was collected after 10 min of centrifugation at 4°C. The luciferase activity of the supernatant was measured with 0.2 µM of luciferin in a luminometer (Perkin Elmer). The protein level was detected with immunoblotting using an α-HA antibody (cat. no. 12013819001, dilution 1:2,000 [v/v]; Roche) and an α-FLAG antibody (cat. no. A8592, dilution 1:2,000 [v/v]; Sigma-Aldrich).

Confocal Microscopy and FLIM-FRET Assays

The FLIM-FRET assay was performed in an Arabidopsis protoplast system. Plasmid DNAs pHB-T-GhLYK5-GBP was co-transformed with pHB-T-GhWAK7A-FLAG or pHB-T-FLAG into protoplasts and incubated for 12 h. The cells were lysed in extraction buffer (100 mM of NaCl, 1 mM of EDTA, 20 mM of Tris-HCl at pH 7.5, 2 mM of NaF, 2 mM of Na₃VO₄, 1 mM of DTT, 0.5% [v/v] Triton X-100, 10% [v/v] glycerol, and 1 × protease inhibitor). The cell lysate was collected after 10 min of centrifugation at 4°C. The luciferase activity of the supernatant was measured with 0.2 µM of luciferin in a luminometer (Perkin Elmer). The protein level was detected with immunoblotting using an α-HA antibody (cat. no. 12013819001, dilution 1:2,000 [v/v]; Roche) and an α-FLAG antibody (cat. no. A8592, dilution 1:2,000 [v/v]; Sigma-Aldrich).

Statistical Analysis

Data for all quantification analyses are presented as mean ± se or sd. The statistical analyses were performed by two-tailed Student’s t test or one-way ANOVA test (*P < 0.05, **P < 0.01). The number of replicates is shown in the figure legends. Statistical results are available in the Supplementary Dataset.

Accession Numbers

Sequence data for the cotton genes described in this article can be found in the databases of G. hirsutum (NAU), G. raimondii (GI), and G. arboreum (CRI; Paterson et al., 2012; Li et al., 2014; Zhang et al., 2015; https://cottonfgd.org/). The accession numbers are listed in Supplemental Tables 6 and 11 and as follows: GhLP2 (Gh_A12G0722), GhMPK3 (Gh_D03G1283), GhWRKY30 (Gh_D12G2738), GhMPK6 (Gh_D02G0105), GhActin9 (Gh_D04G0254), and GhUBQ1 (Gh_A10G0147).

Supplemental Data

Supplemental Figure 1. The phylogeny of cotton WAK with AtWAKs and AtWAKLs.

Supplemental Figure 2. Phylogenetic analysis of WAK proteins from cotton (G. hirsutum, G. raimondii, and G. arboreum), and Arabidopsis.

Supplemental Figure 3. Phylogenetic analysis of WAK proteins from diploid cotton (G. raimondii and G. arboreum), and Arabidopsis.

Supplemental Figure 4. The phylogeny of WAK protein kinase domain sequences.

Supplemental Figure 5. Phylogenetic analysis of A-subgenome GhWAKs with GwWAKs, D-subgenome GwWAKs with GhWAKs, and GaWAKs with GwWAKs.

Supplemental Figure 6. The evolutionary relationship of cotton WAK genes in G. hirsutum, G. raimondii, and G. arboreum.

Supplemental Figure 7. Conserved residues in EGF_like, EGF_Ca²⁺, or WAK_GUB domains of GhWAK proteins.

Supplemental Figure 8. GhWAK7A is not involved in drought or salt stress in cotton.

Supplemental Figure 9. Chitin-induced MAPK activation in VIGS-GhMPK6 and GhMPK3 cotton.

Supplemental Figure 10. Preparation and characterization of OGs and OG-triggered immune responses in cotton.

Supplemental Figure 11. Chitin-induced MAPK activation and ROS production in Arabidopsis lyrata mutants.

Supplemental Figure 12. Phylogenetic analysis of LYK proteins from cotton (G. hirsutum, G. raimondii, and G. arboreum).
Supplemental Figure 13. The conserved amino acid residues of LsyM motif.

Supplemental Figure 14. Genomic locations of GhLYK genes on their corresponding chromosomes of G. hirsutum genome.

Supplemental Figure 15. Phylogenetic analysis of A-subgenome GhLYKs with GaLYKs, D-subgenome GhLYKs with GrLYKs, and GaLYKs with GrLYKs.

Supplemental Figure 16. The evolutionary relationship of cotton LYK genes in G. hirsutum, G. raimondii, and G. arboreum.

Supplemental Figure 17. The VIGS efficiency of VIGS-GhLYK4/5 cotton plants and the specific association of GhLYK5 and GhWAK7A via co-IP and the in vitro phosphorylation assay with GhCERK1CD and GhLYK5CD.

Supplemental Table 1. Conserved motif prediction of WAK proteins from G. hirsutum (NAU assembly).

Supplemental Table 2. Conserved motif prediction of WAK proteins from G. arboreum (CRI assembly).

Supplemental Table 3. Conserved motif prediction of WAK proteins from G. raimondii (JGI assembly).

Supplemental Table 4. Pair-wise protein identity among GhWAKs and AtWAKs.

Supplemental Table 5. Clade partitions of cotton WAKs.

Supplemental Table 6. Gene IDs and the corresponding gene names of GhWAK family.

Supplemental Table 7. Conserved motif prediction of LYK proteins from G. hirsutum (NAU assembly).

Supplemental Table 8. Conserved motif prediction of LYK proteins from G. arboreum (CRI assembly).

Supplemental Table 9. Conserved motif prediction of LYK proteins from G. raimondii (JGI assembly).

Supplemental Table 10. Clade partitions of Arabidopsis and cotton LYKs.

Supplemental Table 11. Gene IDs and the corresponding gene names of the GhLYK family.

Supplemental Table 12. Pair-wise protein identity among GhLYKs and AtLYKs.

Supplemental Table 13. The Di of Vd-inoculated GhLYK-silenced plants from seven repeats.

Supplemental Table 14. The Di of Fov-inoculated GhLYK-silenced plants from six repeats.

Supplemental Table 15. Primers used in this study.

Supplemental Dataset. Statistical results.

Supplemental File 1. Sequence alignments for Figure 1B.

Supplemental File 2. Machine-readable tree file for Figure 1B.

Supplemental File 3. Sequence alignments for Figure 4C.

Supplemental File 4. Machine-readable tree file for Figure 4C.

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AUTHOR CONTRIBUTIONS

P.W., P.H., and L.S. conceived the study and designed the experiments; P.W., L. Zhou, L. Zhang, Z.X.Z., K.B., W.Y.S., L.H.W., P.J., R.M., A.D., and D.P. performed the experiments; P.W., P.J., L. Zhang, I.A., Y.X.H., S.F., P.H., and L.S. analyzed data; P.W., P.J., and L.S. wrote the article; all authors approved the article.

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The Cotton Wall-Associated Kinase GhWAK7A Mediates Responses to Fungal Wilt Pathogens by Complexing with the Chitin Sensory Receptors

Ping Wang, Lin Zhou, Pierce Jamieson, Lin Zhang, Zhixue Zhao, Kevin Babilonia, Wenyong Shao, Lizhu Wu, Roma Mustafa, Imran Amin, Alessandra Diomaiuti, Daniela Pontiggia, Simone Ferrari, Yuxia Hou, Ping He and Libo Shan

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