Identification of Cell Wall-Associated Kinases as Important Regulators Involved in Gossypium Hirsutum Resistance to Verticillium Dahliae

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Abstract

Background

Verticillium wilt, caused by the soil borne fungus *Verticillium dahliae*, is a major threat to cotton production and quality. An increasing number of findings indicate that WAK genes participate in plant–pathogen interactions, but their roles in cotton resistance to *V. dahliae* remains largely unclear.

Results

Here, we carried out a genome-wide analysis of WAK gene family in *Gossypium hirsutum* that resulted in the identification of 81 putative *GhWAKs*, which were all predicted to be localized on plasma membrane. In which, GhWAK77, as a representative, its location was further confirmed using transient expression of fluorescent fusion proteins in tobacco epidermal cells. All GhWAKs could be classified into seven groups according to their various protein domains, indicating that they might sense different outside signals to trigger special intracellular signaling pathways that response to various environmental stresses. A lot of cis-regulatory elements were predicted in the upstream region of *GhWAKs* and classified into four main groups including hormones, biotic, abiotic and light. 31 *GhWAKs*, playing a potential role in the interaction between cotton and *V. dahliae*, were screened out by RNA-seq and qPCR. To further study the function of *GhWAKs* in cotton resistance to *V. dahliae*, VIGS was used to silence *GhWAKs*. At 20 dpi, VIGSed plants exhibited more chlorosis and wilting than the control plants. The disease indices of VIGSed plants were also significantly higher than those of the control. Furthermore, silencing of *GhWAKs* significantly affected the expression of JA- and SA-related marker genes, increased the spread of *V. dahliae* in the cotton stems, dramatically compromised *V. dahliae*-induced accumulation of lignin, H$_2$O$_2$ and NO, but enhanced POD activity.

Conclusion

Our study presents a comprehensive analysis on cotton WAK gene family for the first time. Expression analysis and VIGS provided direct evidences that *GhWAKs* participate in the resistance of cotton to *V. dahliae*.

Background

Tetraploid *Gossypium hirsutum* is the most widely cultivated cotton specie in the world and represents an important source of natural-fiber and oilseed. Verticillium wilt, caused by the soil borne fungus *Verticillium dahliae*, is a major threat to cotton production and quality [1]. Identification and characterization of genes associated with resistance is an important basis for greater understanding on the interaction between cotton and *V. dahliae*, which is necessary for the development of novel disease management methods and new varieties resistant to Verticillium wilt.
Plants live in complex environments crowded with biotic stresses mainly caused by various phytopathogens and pests and exposed to abiotic stresses including cold, hot, drought and salinity. To overcome these stress challenges, plants have evolved a complex and efficient defense signaling network, which include monitoring systems to perceive different stress-derived signals triggering specific defense responses [2]. Cell wall, a dynamic structure surrounding plant cell, has emerged as an essential monitoring system [3, 4]. Some receptor-like kinases (RLKs) have been identified as cell wall integrity sensors that are responsible for the communication between the cell wall and cytoplasm. Typically, plant RLKs contain a signal peptide (SP), transmembrane (TM) domain, and cytoplasmic kinase domain. They can be classified into more than 21 subfamily according to their various extracellular domains [5]. Of which, WAKs (wall-associated kinases) are distinguished from the other RLKs by the presence of their unique extracellular epidermal growth factor (EGF)-like domains [5, 6].

In Arabidopsis thaliana, WAKs are encoded by 5 WAK and 22 WAKL (WAK-like) genes [7]. So far, WAK gene family was also identified in other plants, including Oryza sativa [8], Brassica rapa [9] and Populus trichocarpa [10]. It has been demonstrated that some WAKs are involved in plant development, abiotic and biotic stress responsiveness. Notably, most of WAKs are characterized from Arabidopsis and rice. Arabidopsis AtWAK1, the first identified WAK gene in plant, was shown to contribute to the immune response [11, 12]. A rice WAK gene, OsDEES1 (DEFECT IN EARLY EMBRYO SAC1), plays a role in the regulation of early embryo sac development [13]. OsiWAK1 (O. sativa indica WAK-1) and HvWAK1 (Hordeum vulgare WAK-1) are involved in plant root development [14, 15]. Xa4, encoding a WAK in rice, confers race-specific durable resistance against Xanthomonas oryzae pv. oryzae by reinforcing the cell wall and increasing the production of JA-isoleucine and phytoalexins [16]. OsWAK1 (O. sativa WAK) and OsWAK25, are up-regulated by wounding and salicylic acid (SA), and their overexpression leads to higher resistance in transgenic rice lines against Magnaporthe oryzae [17, 18]. The other four rice WAK genes, including OsWAK14, OsWAK91, OsWAK92 and OsWAK112d, are also suggested to be required for resistance to M. oryzae by loss-of-function mutants [19]. Beyond rice and Arabidopsis, WAKs have been characterized in response to pathogens as well in other plants, such as tomato SlWAK1 (conferring resistance to Pseudomonas syringae) [20], maize ZmWAK (conferring resistance to Sporisorium reilianum) [21] and ZmWAK-RLK1 (conferring resistance to Setosphaeria turcica) [22].

An increasing number of findings indicate that WAK genes participate in plant–pathogen interactions. Therefore, in our study, we used the latest up-land cotton genome sequence data (HAU version 1.1 [23] to explore the WAK gene family, representing the first genome-wide identification of GhWAKs. Moreover, two GhWAKs were functionally characterized in response to V. dahliae infection using VIGS (virus induced gene silencing).

## Results

*G. hirsutum* genome contains 81 *GhWAKs*
The known *A. thaliana* WAK protein sequences were used as queries to search the genome database of *G. hirsutum* accession Texas Marker-1 (TM-1). In total, 81 *GhWAKs* as candidates were identified and named according to their chromosomal locations. These *GhWAKs* were marked on the physical map of 18 chromosomes (Figure 1) and one scaffold664 (*GhWAK65*). A total of 34 and 46 *GhWAKs* were distributed in the A and D sub-genomes, respectively. Chromosome D02 harbored the largest number of *GhWAKs* with 20 genes. Six pairs of tandem duplication events were found, including *GhWAK16/17, GhWAK36/37, GhWAK43/44-49, GhWAK50/52, GhWAK61/62* and *GhWAK69/70/71*. These results revealed that the evolution and expansion of *GhWAKs* happened in *G. hirsutum*, especially on chromosome D02. The detailed information about *GhWAKs*, including gene ID, open reading frame length, amino acid length, protein molecular weight and isoelectric point, instability index and subcellular localization, was listed in Table 1.

All *GhWAKs* were predicted to be localized on plasma membrane (PM) (Table 1). In which, *GhWAK77*, as a representative, its location was further confirmed using transient expression of fluorescent fusion proteins in tobacco epidermal cells. The images clearly showed that fluorescent signal corresponding to the sole *gfp* gene was observed in PM, cytoplasm and nucleus. However, the fluorescent signal corresponding to *GhWAK77-gfp* was solely shown in PM (Figure 2). These suggest that *GhWAKs* have potential as connector responsible for communication between inside and outside of the cell.

**GhWAKs have conservative kinase domains and various extracellular domains**

The majority of *GhWAKs* have 3-4 introns and show similar exon-intron structure (Figure 3A). *GhWAK36* contains the maximum number introns (six), whereas *GhWAK11* and *GhWAK53* do not contain any intron. A total of six conserved protein domains were identified in *GhWAKs*, including GUB_WAK_bind (wall-associated receptor kinase galacturonan-binding, PF13947), WAK (wall-associated kinase, PF08488), WAK assoc (wall-associated receptor kinase C-terminal, PF14380), EGF (EGF, PF00008; cEGF, PF12662; hEGF, PF12661; EGF_CA, PF07645; EGF_3, PF12947), DUF1199 (domain of unknown function, PF06712) and protein kinase domain (pkinase, PF00069; pkinase_Tyr, PF07714; kinase-like, PF14531; protein-kinase domain of FAM69, PF12260) (Figure 3B). Cytoplasmic, extracellular and TM regions were predicted in the majority of *GhWAKs*, further indicating that they are membrane proteins. Typical WAK encodes a transmembrane protein with a cytoplasmic kinase domain and an extracellular region. However, several proteins showed uncommon structural characteristics, such as the kinase domain in extracellular region (*GhWAK43, GhWAK54, GhWAK75, GhWAK57, GhWAK2, GhWAK36, GhWAK37 and GhWAK40*), double TMs (*GhWAK18, GhWAK58, GhWAK59, GhWAK60, GhWAK64, GhWAK75 and GhWAK1*) and kinase domains (*GhWAK59*). All *GhWAKs* were classified into seven groups according to their protein domain analysis (Figure 3B and 3C). The members in Group I, Group II and Group III were typical WAKs that contain EGF domain in extracellular region. The other four Groups, including IV, V, VI and VII, do not contain EGF. *GhWAKs* in Group I and IV contain both WAK and GUB domain. Inversely, *GhWAKs* in Group III neither contain WAK nor GUB domain. *GhWAKs* in Group II, VI and VII only contain GUB domain. However, II and VI are one-GUB-domain groups, and VII are two-GUB-domain group. *GhWAKs* in Group V only contain WAK domain. In additionally, DUF1199 domain was found in *GhWAK31*.
and GhWAK77 (Figure 3B). Different types and numbers of extracellular domains were present in GhWAKs, indicating that they might sense or bind different outside signaling to trigger special intracellular signaling pathways that control plant development and responses to various environmental stresses.

**Prediction of putative cis-regulatory elements in GhWAK promoters**

The 2 kb region upstream of the translation start site of all GhWAKs were considered the promoter and analyzed for investigating the potential roles of cis-regulatory elements. The numbers and names of identified cis-elements were shown on Figure 4. These cis-regulatory elements were classified into four main groups including hormones, biotic, abiotic and light. Twelve hormone-responsive regulatory elements associated with abscisic acid (ABA) (ABRE, ABRE4 and AT-ABRE), auxin (IAA) (AuxRR-core, TGA-box and TGA-element), methyl jasmonate (MeJA) (CGTCA-motif), gibberellin (GA) (GARE-motif, P-box and TATC-box), SA (TCA-element) and ethylene (ET) (ERE), were identified. Of which, ABRE-motif, CGTCA-motif and ERE were enriched in the most of GhWAK promoters, indicating that they might be widely induced by ABA, JA and ET. The biotic stress-related regulatory elements, such as AT-rich, TC-rich repeats, W-box, WUN-motif, WRE3, JERE and box S, were involved in elicitor-mediated activation, wounding and pathogen responsiveness. In additionally, eight abiotic-responsive regulatory elements, associated with anaerobic induction (ARE and GC-motif), low-temperature responsiveness (LTR), drought-inducibility (MBS, DRE core and DRE1), heat shock, osmotic stress, low pH, nutrient starvation (STRE) and stress-related (TCA), were identified in the GhWAK promoter regions. Moreover, various light-responsive elements were present in the promoters of GhWAKs. Especially, Box 4 and G-Box were widely harbored. These results indicated that GhWAKs might play vital roles in the response to various stresses, hormones and light.

**GhWAKs were significantly induced by V. dahliae infection**

To identify GhWAKs that are related to V. dahliae infection, two-fold changes were applied in transcript expression profiles from RNA-seq as minimum cutoffs. As a result, 26 GhWAKs were screened out, including 17 up-regulated and 9 down-regulated genes (Figure 5). Of these, 11 GhWAKs, including GhWAK5, GhWAK9, GhWAK77, GhWAK10, GhWAK45, GhWAK47, GhWAK78, GhWAK48, GhWAK31, GhWAK26 and GhWAK72, were significantly up-regulated in at least three time points. Further, the expression profiles of GhWAKs in response to the infection with V. dahliae were detected and verified through qRT-PCR (Figure 6). GhWAK1 and GhWAK69 showing higher transcription levels in roots of cotton seedlings inoculated with V. dahliae than control was screened out. Due to the high degree of sequence similarity in GhWAKs family, it is difficult to design specific primers for four gene pairs, including GhWAK4 & GhWAK45, GhWAK5 & GhWAK49, GhWAK10 &GhWAK55, and GhWAK31 & GhWAK77. The results of qRT-PCR indicated that these four pairs of genes were dramatically up-regulated. According to RNA-seq data, GhWAK4, GhWAK49 and GhWAK55 did not shown to be up-regulated. Thus, the expression changes found using qRT-PCR probably more represent the responses of GhWAK45, GhWAK5 and GhWAK10 to V. dahliae infection. The expression results of GhWAK26,
GhWAK48 and GhWAK72 were consistent with RNA-seq data with up-regulation. In total, 31 GhWAKs were screened out, which play a potential role in the interaction between cotton and V. dahliae.

**Silencing of GhWAKs compromises cotton resistance to Verticillium wilt**

GhWAK26 and GhWAK77 showed obviously persistent up-regulated expression to the infection from V. dahliae (Figure 5 and Figure 6). In addition, they contain cis-elements in their promoters associated with MeJA and SA, which play key roles in cotton resistance to V. dahliae. Thus, to further reveal the function of GhWAKs in cotton resistance to V. dahliae, GhWAK26 and GhWAK77 were prioritized for study as representatives in this study using TRV based VIGS system. At approximately two weeks post-infiltration with a mixture of Agrobacterium cultures containing pTRV1 and pTRV2-CLA1, a strong photobleaching phenotype was shown on the newly emerging true leaves (Figure 7A), indicating that VIGS system worked well. Then, the expression levels of GhWAK26 and GhWAK77 were detected in the leaves infiltrated with pTRV2-GhWAK26 and pTRV2-GhWAK77, respectively. As shown in Figure 7B, the expression levels of GhWAK26 and GhWAK77 were reduced by about 80%, suggesting VIGS triggered their silencing in cotton plants. At 20 dpi, VIGSed plants (Figure 7D and 7E) exhibited more chlorosis and wilting than the control plants infiltrated with Agrobacterium cultures containing empty vector pTRV1 and pTRV2 (Figure 7C). The disease indices of VIGSed plants were also significantly higher than those of the control at 15 dpi and 20 dpi (Figure 7F). Therefore, the results of VIGS assays suggested that GhWAK26 and GhWAK77 are important participants in cotton resistance to V. dahliae infection.

**Silencing of GhWAKs significantly affected the expression of JA and SA-related marker genes**

Further, the expression of several JA and SA-related marker genes involved in plant defense signaling pathways was detected. The expression of JAZ1 (jasmonate-zim-domain protein), JAZ3, JAZ6, LOX1 (lipoxygenase) (JA-related marker genes), PR3 (pathogenesis related protein) and NPR1 (nonexpresser of PR protein) (SA-related marker genes) were significantly down-regulated after silencing of GhWAK26 in cotton (Figure 8A). In GhWAK77-silenced plants, JAZ6 and three important genes involved in the SA signaling pathway, including ICS1 (isochorismate synthase), NPR1 and EDS1 (enhanced disease susceptibility), were down-regulated comparing with control. On the contrary, the expression of JAZ1 and LOX1 were significantly up-regulated due to the silencing of GhWAK77 (Figure 8B). These indicated that GhWAK26 and GhWAK77 may involve in cotton resistance to V. dahliae by SA and JA signaling passway.

**Silencing of GhWAKs increased the spread of V. dahliae in the cotton stems**

After inoculation, V. dahliae in cotton stems was detected by PCR. As shown in Figure 9A, no specific amplification products from V. dahliae were shown in CK at 5 dpi and 7 dpi, indicating that V. dahliae had not yet invaded the stems or multiplied in large quantities. However, at 5 dpi, few specific products from V. dahliae were amplified in GhWAK26-silenced and GhWAK77-silenced plants, representing a small amount of pathogen invasion. Further, at 7 dpi, the bright bands amplified using stems from GhWAK26-silenced and GhWAK77-silenced plants appeared on agarose gels, indicating that V. dahliae had invaded largely. In addition, pathogen isolation on PDA showed that a large number of V. dahliae grew out from the stems of
GhWAK26-silenced and GhWAK77-silenced cotton plants, while no mycelium was shown from the control (Figure 9B). Both PCR detection and PDA culture results suggested that silencing GhWAKs significantly increased the spread of V. dahliae in the cotton stems.

Lignin is considered to play an important role in preventing the infection of V. dahliae in cotton. Therefore, we further compared the changes of lignin content in GhWAK-silenced cotton stems with CK. The results showed that the lignin content in GhWAK-silenced plants was significantly lower than that in CK (Figure 9C), which might affect the stem structure and then reduce the prevention of cotton on V. dahliae infection.

**Silencing of GhWAKs dramatically compromised V. dahliae-induced accumulation of H$_2$O$_2$ and NO (nitric oxide), but enhanced POD (peroxidase) activity**

The contents of H$_2$O$_2$ and NO, and POD activity in GhWAK-silenced plants inoculated with V. dahliae were further measured. GhWAKs silencing caused lower levels of H$_2$O$_2$ at 6 hpi (hours post inoculation), 12 hpi and 24 hpi (Figure 10A and 10B). Both GhWAK26- and GhWAK77-silenced plants accumulated greatly depressed levels of NO comparing with CK (Figure 10C and 10D). However, the activity of POD significantly elevated in GhWAK26- and GhWAK77-silenced plants at 6 hpi, 24 hpi and 48 hpi, except at 12 hpi (Figure 10E and 10F).

**Discussion**

WAK gene family has been analyzed in several plant species, such as A. thaliana [7], O. sativa [8], P. trichocarpa [10] and B. rapa [9]. Some WAKs have been implicated in the response to pathogenic infection. Examples are Arabidopsis Wak1 [12], maize ZmWAK-RLK1 (Htn1) and ZmWAK (qHSR1) [21, 22], wheat Stb6 and TaWAK6 [24, 25], rice Xa4, OsWAK1 and OsWAK91 [16, 17, 26], and orange CsWAKL08 [27], which confer host plant disease resistance. Here, a total of 81 GhWAKs were systematically identified and analyzed for the first time from a high-quality G. hirsutum genome (Table 1), which was assembled by integrating single-molecule real-time sequencing, BioNano optical mapping and high-throughput chromosome conformation capture techniques [23]. Of which, 31 GhWAKs were potentially involved into the interaction between cotton and V. dahliae (Figure 5 and Figure 6). Especially, silencing of GhWAK26 or GhWAK77 dramatically reduced the resistance of cotton plants to V. dahliae infection (Figure 7), suggesting that WAKs are important resistance genes during cotton–pathogen interactions.

At the PM, RLKs as cell-surface receptors can perceive and process extracellular danger signals to trigger plant defense responses [28]. WAK is part of RLK subfamilies. All GhWAKs contain a typical eukaryotic kinase domain that is mostly present in intracellular region and relatively well conserved (Figure 3B). In addition, GhWAKs locate on PMs in all probability (Table 1, Figure 2), suggesting that GhWAKs have potential roles in communicating between inside and outside of the cell. In order to penetrate plant roots to gain access to the xylem and to spread in the vascular system, V. dahliae usually secretes various
toxins and carbohydrate active enzymes, including glycoproteins and cell wall-degrading enzymes [29, 30]. Therefore, it is conceivable that *V. dahliae* infection would affect plant cell wall integrity (CWI) and generate some degradation products, which are important defense signals [31]. In the extracellular region, GhWAKs contain five different domains (Figure 3B), which may sense CWI or interact with different components of these extracellular matrix, such as glycine-rich protein, pectin and OGs [32-34].

At present, the molecular mechanisms of *WAK*-mediated resistance remain largely unknown. However, some defence responses associated with WAKs have been reported, including cell wall reinforcement [16], pathogenesis-related genes activation [18], SA or JA accumulation [27], POD and superoxide dismutase activities [27], and reactive oxygen species (ROS) homeostasis [27]. Here, silencing of *GhWAKs* resulted in the up- or down-regulation of several genes (Figure 8) and depressed cotton resistance to *V. dahliae*. Among them, *JAZ* and *LOX* are associated with JA-mediated defense responses [35]. *NPR1, ICS1* and *EDS1* are associated with SA-mediated defense responses [36]. These two phytohormones, JA and SA, have been known to be involved into the regulation of plant resistance against *V. dahliae* [37, 38]. In addition, some hormone-responsive (including JA and SA) and biotic stress-related regulatory elements were enriched in the promoters of *GhWAKs* (Figure 4). Thus, these findings suggest that *GhWAK* function as a mediator to active intracellular SA and JA signaling pathways to regulate plant resistance.

*V. dahliae* is a vascular pathogen that penetrates the host roots and then extends to other parts of the overground parts of plant through the process of transpiration [29, 37]. The improvement of physical, chemical and structural barriers, such as ROS, NO, cell wall, lignin, callose and POD, contributes to preventing expansion and reducing colonization of *V. dahliae* in cotton tissues [37, 39-41]. In this study, more *V. dahliae* was detected in *GhWAK26*-silenced or *GhWAK77*-silenced plants with lower lignin contents than in CK (Figure 9). Moreover, silencing of *GhWAKs* in cotton plants dramatically compromised *V. dahliae*-induced accumulation of H$_2$O$_2$ and NO, but enhanced POD activity (Figure 10). These findings demonstrate that *GhWAKs* play roles in preventing pathogen spreading at least in part by regulating the accumulation of lignin, H$_2$O$_2$ and NO, and the activity of POD. Overall, our study augments our knowledge about cotton WAK gene family, and particularly promotes the understanding their function in disease resistance.

**Conclusions**

In this study, we carried out a genome-wide analysis of WAK gene family in *G. hirsutum* that resulted in the identification of 81 putative *GhWAKs*, which might sense different outside signals to trigger special intracellular signaling pathways that response to various environmental stresses. Of which, 31 *GhWAKs* with potential roles in the interaction between cotton and *V. dahliae* were screened out. Silencing of *GhWAKs* could significantly affected the expression of JA- and SA-related marker genes, increased the spread of *V. dahliae* in the cotton stems, dramatically compromised *V. dahliae*-induced accumulation of lignin, H$_2$O$_2$ and NO, but enhanced POD activity. These results provided direct evidences that *GhWAKs* participate in the resistance of cotton to *V. dahliae*.
Methods

Identification and bioinformatics analysis of GhWAKs

The amino acid and nucleotide sequences of WAKs from Arabidopsis accessed from TAIR website (https://www.Arabidopsis.org/) were queried against *G. hirsutum* genome database (HAU) in CottonFGD (https://cottonfgd.org/) using BLAST program (E-value < 0.01) [7, 23]. The obtained putative protein sequences of GhWAKs were further identified by HMMER software (HMM Database = Pfam; Significance E-values < 0.01) (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) to confirm the presence of conserved protein domains.

Functional sites and transmembrane topology for all putative GhWAKs were analyzed through PROSITE database (https://prosite.expasy.org/) and Phobius database (http://phobius.sbc.su.se/), respectively. The number of amino acids, molecular weight, theoretical isoelectric point and instability index of proteins were analyzed using ExPASy program (http://www.expasy.org/). Prediction of protein subcellular localization were performed using CELLO v2.5 (http://cello.life.nctu.edu.tw/) and ProtComp 9.0 (http://www.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc). Signal peptides were predicted using SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/).

Analysis of chromosomal location, genes structure and cis-elements

The information about physical chromosomal locations and gene structures of GhWAKs were extracted from *G. hirsutum* genome database [23] and analyzed by TBtools software [42]. The potential promoter sequences, 2 kb upstream of GhWAKs, were also extracted from *G. hirsutum* genome database. The cis-elements in the potential promoters were predicted in PlantCARE databases (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Plant materials and *V. dahliae* inoculation

The seeds of *Nicotiana benthamiana* and *G. hirsutum* cv. Nongda 601 (ND601) were preserved at the State Key Laboratory of North China Crop Improvement and Regulation, Hebei Agricultural University, China. *N. benthamiana* was grown in the greenhouse about 5 weeks at 21°C with 14/10 h (light/dark) photoperiod. *G. hirsutum* cv. ND601 were grown in the greenhouse at 25°C under a 14-h light/10-h dark cycle with relative humidity about 70%. *V. dahliae* (strain Linxi 2-1) cultivation and inoculation with cotton seedlings by soil drenching were described previously [39], using 30 mL of spore suspension (10⁷ spores ml⁻¹).

Proteins subcellular localization

The ORF of *GhWAK77* (without the stop codon) was amplified by PCR with primers gWAK77-F and gWAK77-R (Table S1), then introduced into entry vector pDONR™207 by BP reaction, as described by the manufacturer (Invitrogen). The *GhWAK77* fragment was transferred from the entry clone to expression vector pEarlyGate103 [43] with LR recombinant reaction, as described by the manufacturer (Invitrogen).
The recombinant expression vector was introduced into Agrobacterium tumefaciens GV3101, cultured and infiltrated into four-week-old tobacco leaves via the method described by [44]. After 2 days, GFP signal in the tobacco leaf epidermal cell was examined using a laser scanning microscope (FluoView FV1000; Olympus).

**RNA-seq data and qPCR analysis**

The transcription patterns of GhWAKs in cotton roots after inoculation with V. dahliae were analyzed using high-through RNA-seq data published previously [37]. Log₂^{Fold change} were calculated from FPKM (fragments per kilobase of exon model per million mapped) and used for the heat map of hierarchical clustering with the TBtools v0.67 software [42]. Total RNA was extracted using EASYspin Plant RNA kit (Aidlab, Beijing, China) according to the manufacturer’s instructions. The quality and concentration of RNA were detected by 1.5% agarose gel electrophoresis and NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific), respectively. cDNA was synthesized with a reverse transcription kit (ReverTra Ace® qPCR RT Master Mix with gDNA Remover, TaKaRa, Dalian, China). qPCR was performed using 7500 Real Time PCR System (Applied Biosystems, USA) with THUNDERBIRD®SYBR® qPCR Mix (TaKaRa, Dalian, China). The 2^{-ΔΔCt} method was used to calculate the relative expression of genes. GhHis 3 was used as internal reference. Three biological repeats were taken for each treatment.

**VIGS assays in cotton**

The vectors for VIGS, pTRV1 and pTRV2, were kindly provided by Professor Liu Yule of Tsinghua University [45]. The fragments from GhWAKs were amplificated by PCR and inserted into the pTRV2 vector between EcoRI and KpnI. The constructed vectors were separately transferred into A. tumefaciens strain GV3101 by freeze-thaw method [46]. VIGS in cotton was performed as described previously [47]. At least 30 plants were used per treatment, and each treatment was repeated three times. Plant resistance to V. dahliae was assayed using disease index [48].

**Detection and isolation of V. dahliae in cotton stems**

At 5 dpi and 7 dpi, 1 cm and 0.5 cm of samples excised at a height of 0.5 cm stem above ground were used for detection and isolation of V. dahliae, respectively. V. dahliae detection by PCR was performed using primers P1 and P2 [49]. V. dahliae isolation from cotton stems was carried out according to the previous method [50]. Twenty-four individual plants were sampled for each treatment and repeated thrice.

**Measurements of NO, H₂O₂ and POD activity**

The first true leaves of cotton seedlings were powdered in the mortar with liquid nitrogen and homogenized using 50 mM sodium phosphate buffer (pH 7.0). After centrifugation (14000 g, 20 min), the supernatants were used for the determination of NO, H₂O₂ and POD activity with commercialized assay kits (Nanjing Jiancheng Bioengineering Institute, China), following the manuals. The total protein concentration of the supernatant was measured using Pierce™ BCA Protein Assay Kit (Thermo Scientific).
**Primers and statistical analysis**

All primers used in this study were listed in Table S1. Differences between measured values were analyzed using software GraphPad Prism® 8 (GraphPad, San Diego, CA, USA). The $P$-value less than 0.05 was assumed to be statistically significant.

**Abbreviations**

ABA: abscisic acid; CWI: cell wall integrity; EDS: enhanced disease susceptibility; EGF: epidermal growth factor; ET: ethylene; FPKM: fragments per kilobase of exon model per million mapped; GA: gibberellin; hpi: hours post inoculation; ICS: isochorismate synthase; JAZ: jasmonate-zim-domain protein; LOX: lipoxygenase; MeJA: methyl jasmonate; NO: nitric oxide; NPR: nonexpresser of PR protein; PM: plasma membrane; POD: peroxidase; PR: pathogenesis related protein; RLKs: receptor-like kinases; ROS: reactive oxygen species; SA: salicylic acid; SP: signal peptide; TM: transmembrane; VIGS: virus-induced gene silencing; WAKs: wall-associated kinases.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no conflict of interest.

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**Availability of data and materials**

The data generated or analyzed during the current study are included in this published article and its supplemental data files and available from the corresponding author on reasonable request.

**Authors’ contributions**
YJ, WXF and MZY. designed the experiments and wrote the manuscript. YJ, XMX and WGN performed most of the experiments. The other authors assisted in the experiments, analyzed the data and discussed the results. All authors read and approved the manuscript.

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**Tables**

**Table 1** Detailed information of the putative upland cotton WAK genes identified in this study
| Gene Name | Gene ID    | ORF (bp) | Length (aa) | MW (kDa) | pI   | Instability index | Subcellular localization |
|-----------|------------|----------|-------------|----------|------|-------------------|--------------------------|
| GhWAK1    | Ghir_A02G001840 | 2217     | 738         | 82.81    | 6.86 | 42.46             | PM                       |
| GhWAK2    | Ghir_A02G001850 | 2880     | 959         | 107.41   | 5.97 | 41.51             | PM                       |
| GhWAK3    | Ghir_A02G002660 | 2283     | 760         | 85.46    | 5.56 | 39.76             | PM                       |
| GhWAK4    | Ghir_A02G007280 | 2178     | 725         | 80.83    | 5.61 | 33.14             | PM                       |
| GhWAK5    | Ghir_A02G007310 | 2256     | 751         | 83.41    | 6.17 | 35.88             | PM                       |
| GhWAK6    | Ghir_A02G007330 | 2232     | 743         | 82.94    | 5.91 | 39.16             | PM                       |
| GhWAK7    | Ghir_A02G007350 | 2121     | 706         | 78.95    | 6.80 | 31.85             | PM                       |
| GhWAK8    | Ghir_A02G012070 | 2190     | 729         | 81.41    | 5.80 | 38.89             | PM                       |
| GhWAK9    | Ghir_A02G012080 | 2229     | 742         | 81.19    | 5.33 | 35.72             | PM                       |
| GhWAK10   | Ghir_A02G017660 | 2103     | 700         | 77.18    | 8.56 | 40.12             | PM                       |
| GhWAK11   | Ghir_A03G016250 | 1908     | 635         | 70.57    | 6.72 | 41.61             | PM                       |
| GhWAK12   | Ghir_A03G016560 | 2025     | 674         | 75.73    | 6.20 | 47.28             | PM                       |
| GhWAK13   | Ghir_A04G009230 | 1905     | 634         | 70.44    | 8.62 | 33.66             | PM                       |
| GhWAK14   | Ghir_A05G020230 | 2073     | 690         | 76.18    | 6.65 | 44.81             | PM                       |
| GhWAK15   | Ghir_A05G024460 | 2085     | 694         | 76.91    | 5.15 | 32.81             | PM                       |
| GhWAK16   | Ghir_A05G024500 | 2094     | 697         | 77.68    | 5.53 | 39.57             | PM                       |
| GhWAK17   | Ghir_A05G024510 | 2130     | 709         | 79.13    | 8.35 | 36.54             | PM                       |
| GhWAK18   | Ghir_A06G001260 | 2103     | 700         | 77.81    | 7.73 | 49.35             | PM                       |
| GhWAK19   | Ghir_A09G001860 | 2844     | 947         | 106.41   | 7.60 | 44.97             | PM                       |
| GhWAK20   | Ghir_A09G005720 | 1923     | 640         | 72.11    | 6.47 | 38.48             | PM                       |
| GhWAK21   | Ghir_A09G016250 | 1923     | 640         | 71.10    | 8.79 | 34.18             | PM                       |
| GhWAK22   | Ghir_A10G009180 | 2082     | 693         | 76.58    | 8.48 | 45.50             | PM                       |
| GhWAK23   | Ghir_A10G013470 | 2889     | 962         | 107.19   | 6.20 | 47.17             | PM                       |
| GhWAK24   | Ghir_A10G018760 | 2058     | 685         | 76.72    | 6.37 | 36.17             | PM                       |
| GhWAK25   | Ghir_A10G019250 | 2253     | 750         | 83.65    | 5.99 | 38.92             | PM                       |
| GhWAK26   | Ghir_A10G022760 | 1890     | 629         | 68.77    | 6.35 | 40.73             | PM                       |
| GhWAK27   | Ghir_A11G011010 | 1848     | 615         | 67.03    | 5.65 | 39.17             | PM                       |
| Location   | Name     | Year  | Number | Longitude | Latitude | Temperature    | Humidity   | Pressure    | Wind Speed | Wind Direction | Time of Day |
|------------|----------|-------|--------|-----------|----------|---------------|------------|------------|------------|---------------|-------------|
| GhWAK28    | Ghir_A11G015050 | 2085  | 694    | 78.23     | 6.43     | 38.74         | PM         |            |            |               |             |
| GhWAK29    | Ghir_A11G017400 | 1965  | 654    | 74.59     | 8.76     | 36.61         | PM         |            |            |               |             |
| GhWAK30    | Ghir_A11G017530 | 2007  | 668    | 75.17     | 6.40     | 36.25         | PM         |            |            |               |             |
| GhWAK31    | Ghir_A11G019930 | 2091  | 696    | 76.73     | 7.15     | 45.22         | PM         |            |            |               |             |
| GhWAK32    | Ghir_A11G026030 | 1857  | 618    | 69.06     | 8.98     | 35.75         | PM         |            |            |               |             |
| GhWAK33    | Ghir_A12G005550 | 1953  | 650    | 72.70     | 5.17     | 46.76         | PM         |            |            |               |             |
| GhWAK34    | Ghir_A12G012670 | 1890  | 629    | 69.54     | 6.26     | 37.01         | PM         |            |            |               |             |
| GhWAK35    | Ghir_D02G001920 | 2805  | 934    | 104.86    | 5.47     | 43.66         | PM         |            |            |               |             |
| GhWAK36    | Ghir_D02G001930 | 2736  | 911    | 101.87    | 6.09     | 48.28         | PM         |            |            |               |             |
| GhWAK37    | Ghir_D02G001940 | 2766  | 921    | 102.98    | 6.15     | 47.44         | PM         |            |            |               |             |
| GhWAK38    | Ghir_D02G001960 | 2877  | 958    | 107.08    | 7.72     | 40.71         | PM         |            |            |               |             |
| GhWAK39    | Ghir_D02G001970 | 3015  | 1004   | 112.34    | 7.20     | 43.96         | PM         |            |            |               |             |
| GhWAK40    | Ghir_D02G001980 | 2853  | 950    | 105.62    | 5.97     | 40.01         | PM         |            |            |               |             |
| GhWAK41    | Ghir_D02G003070 | 2925  | 974    | 109.39    | 5.69     | 39.50         | PM         |            |            |               |             |
| GhWAK42    | Ghir_D02G007710 | 1929  | 642    | 71.21     | 6.81     | 39.67         | PM         |            |            |               |             |
| GhWAK43    | Ghir_D02G007720 | 2052  | 683    | 75.70     | 5.31     | 38.92         | PM         |            |            |               |             |
| GhWAK44    | Ghir_D02G007730 | 2238  | 745    | 82.72     | 5.20     | 40.76         | PM         |            |            |               |             |
| GhWAK45    | Ghir_D02G007740 | 2253  | 750    | 84.49     | 6.17     | 38.33         | PM         |            |            |               |             |
| GhWAK46    | Ghir_D02G007750 | 2196  | 731    | 81.89     | 5.98     | 37.64         | PM         |            |            |               |             |
| GhWAK47    | Ghir_D02G007760 | 2049  | 682    | 75.75     | 5.36     | 36.84         | PM         |            |            |               |             |
| GhWAK48    | Ghir_D02G007780 | 2313  | 770    | 85.69     | 6.12     | 36.93         | PM         |            |            |               |             |
| GhWAK49    | Ghir_D02G007790 | 2214  | 737    | 81.56     | 5.98     | 37.05         | PM         |            |            |               |             |
| GhWAK50    | Ghir_D02G007800 | 1905  | 634    | 71.16     | 5.77     | 33.20         | PM         |            |            |               |             |
| GhWAK51    | Ghir_D02G007810 | 2163  | 720    | 80.85     | 6.13     | 41.17         | PM         |            |            |               |             |
| GhWAK52    | Ghir_D02G007820 | 2151  | 716    | 80.23     | 6.54     | 35.66         | PM         |            |            |               |             |
| GhWAK53    | Ghir_D02G017510 | 1908  | 635    | 70.61     | 6.55     | 44.29         | PM         |            |            |               |             |
| GhWAK54    | Ghir_D02G017820 | 1890  | 629    | 70.82     | 6.12     | 48.70         | Ex, PM     |            |            |               |             |
| GhWAK55    | Ghir_D03G001900 | 2100  | 699    | 76.95     | 8.54     | 38.15         | PM         |            |            |               |             |
| GhWAK56    | Ghir_D03G011850 | 2076  | 691    | 76.83     | 8.55     | 33.01         | PM         |            |            |               |             |
| GhWAK57     | Ghir_D04G013370 | 1920 | 639 | 71.07 | 8.58 | 32.21 | PM  |
|-------------|-----------------|------|-----|-------|------|-------|-----|
| GhWAK58     | Ghir_D05G020210 | 2181 | 726 | 80.64 | 7.19 | 45.94 | PM  |
| GhWAK59     | Ghir_D05G024300 | 3069 | 1022 | 114.00 | 6.47 | 33.32 | PM  |
| GhWAK60     | Ghir_D06G001130 | 2103 | 700 | 77.76 | 7.74 | 47.04 | PM  |
| GhWAK61     | Ghir_D09G001670 | 2862 | 953 | 106.48 | 6.04 | 42.26 | PM  |
| GhWAK62     | Ghir_D09G001690 | 2850 | 949 | 106.27 | 5.71 | 43.80 | PM  |
| GhWAK63     | Ghir_D09G015720 | 1914 | 637 | 70.65 | 8.74 | 35.45 | PM  |
| GhWAK64     | Ghir_D09G018010 | 1995 | 664 | 75.49 | 6.23 | 42.60 | PM  |
| GhWAK65     | Ghir_D09G025850 | 1971 | 656 | 74.68 | 6.20 | 43.00 | PM  |
| GhWAK66     | Ghir_D10G010060 | 2082 | 693 | 76.31 | 8.53 | 46.47 | PM  |
| GhWAK67     | Ghir_D10G014200 | 2898 | 965 | 107.43 | 5.62 | 47.03 | PM  |
| GhWAK68     | Ghir_D10G020270 | 2049 | 682 | 76.45 | 6.53 | 35.60 | PM  |
| GhWAK69     | Ghir_D10G020870 | 2091 | 696 | 77.38 | 6.38 | 34.71 | PM  |
| GhWAK70     | Ghir_D10G020880 | 2310 | 769 | 86.29 | 6.24 | 34.86 | PM  |
| GhWAK71     | Ghir_D10G020930 | 2307 | 768 | 86.09 | 5.67 | 35.08 | PM  |
| GhWAK72     | Ghir_D10G025210 | 1830 | 609 | 66.92 | 6.20 | 45.83 | PM  |
| GhWAK73     | Ghir_D11G010940 | 1923 | 640 | 69.64 | 5.89 | 40.12 | PM  |
| GhWAK74     | Ghir_D11G015120 | 1902 | 633 | 71.17 | 6.12 | 44.54 | PM  |
| GhWAK75     | Ghir_D11G017450 | 1992 | 663 | 74.61 | 8.03 | 50.47 | PM  |
| GhWAK76     | Ghir_D11G017550 | 2058 | 685 | 75.91 | 5.97 | 47.03 | PM  |
| GhWAK77     | Ghir_D11G020010 | 2094 | 697 | 76.74 | 6.92 | 46.71 | PM  |
| GhWAK78     | Ghir_D11G023010 | 2040 | 679 | 75.99 | 6.72 | 36.11 | PM  |
| GhWAK79     | Ghir_D11G026200 | 1908 | 635 | 70.99 | 8.78 | 38.79 | PM  |
| GhWAK80     | Ghir_D12G005550 | 2004 | 667 | 74.32 | 5.50 | 46.69 | PM  |
| GhWAK81     | Ghir_D12G012920 | 1896 | 631 | 69.95 | 6.55 | 36.83 | PM  |

PM, plasma membrane; Ex, extracellular;