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 predicated 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_2O_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 predicated 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 haveconservative 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 predicated 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* & *GhWAK*49, *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*, *GhWAK*49 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_2O_2 and NO (nitric oxide), but enhanced POD (peroxidase) activity

The contents of H_2O_2 and NO, and POD activity in *GhWAK*-silenced plants inoculated with *V. dahliae* were further measured. *GhWAKs* silencing caused lower levels of H_2O_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_2O_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_2O_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_2O_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℃ with 14/10 h (light/dark) photoperiod. *G. hirsutum* cv. ND601 were grown in the greenhouse at 25℃ 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 (10^7 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^{TM}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]. $Log_2^{Fold\ change}$ 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 $2^{-\Delta\Delta Ct}$ 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 *EcoR* I and *Kpn* I. 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, H_2O_2 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, H_2O_2 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

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;

Figures

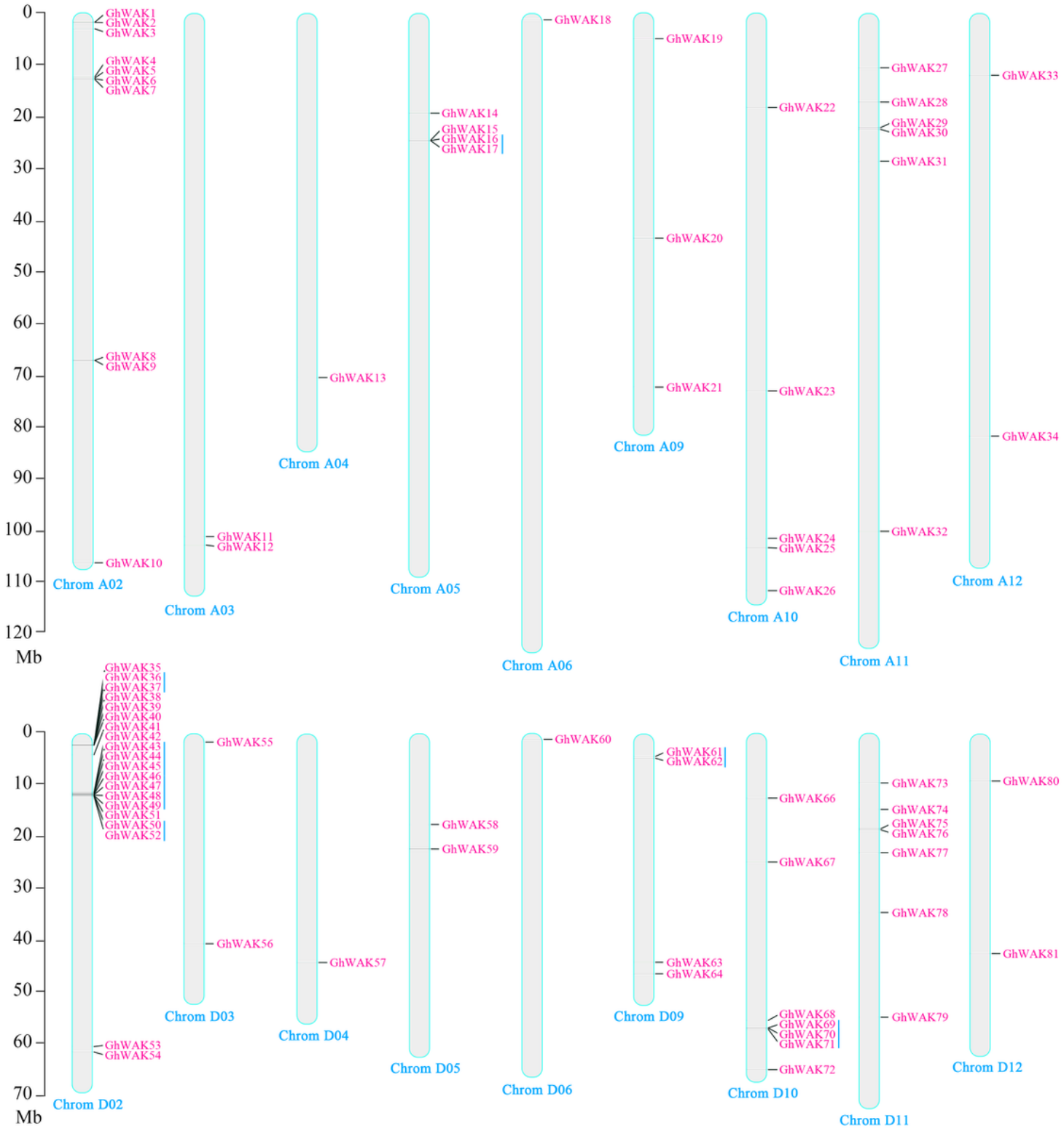

Figure 1

Chromosomal distributions of GhWAKs in G. hirsutum. The chromosomal positions of GhWAKs were mapped according to the up-land cotton genome. The scale is in mega bases (Mb). The chromosome number was indicated at the bottom of each chromosome. Tandem duplicated genes were marked with blue line.

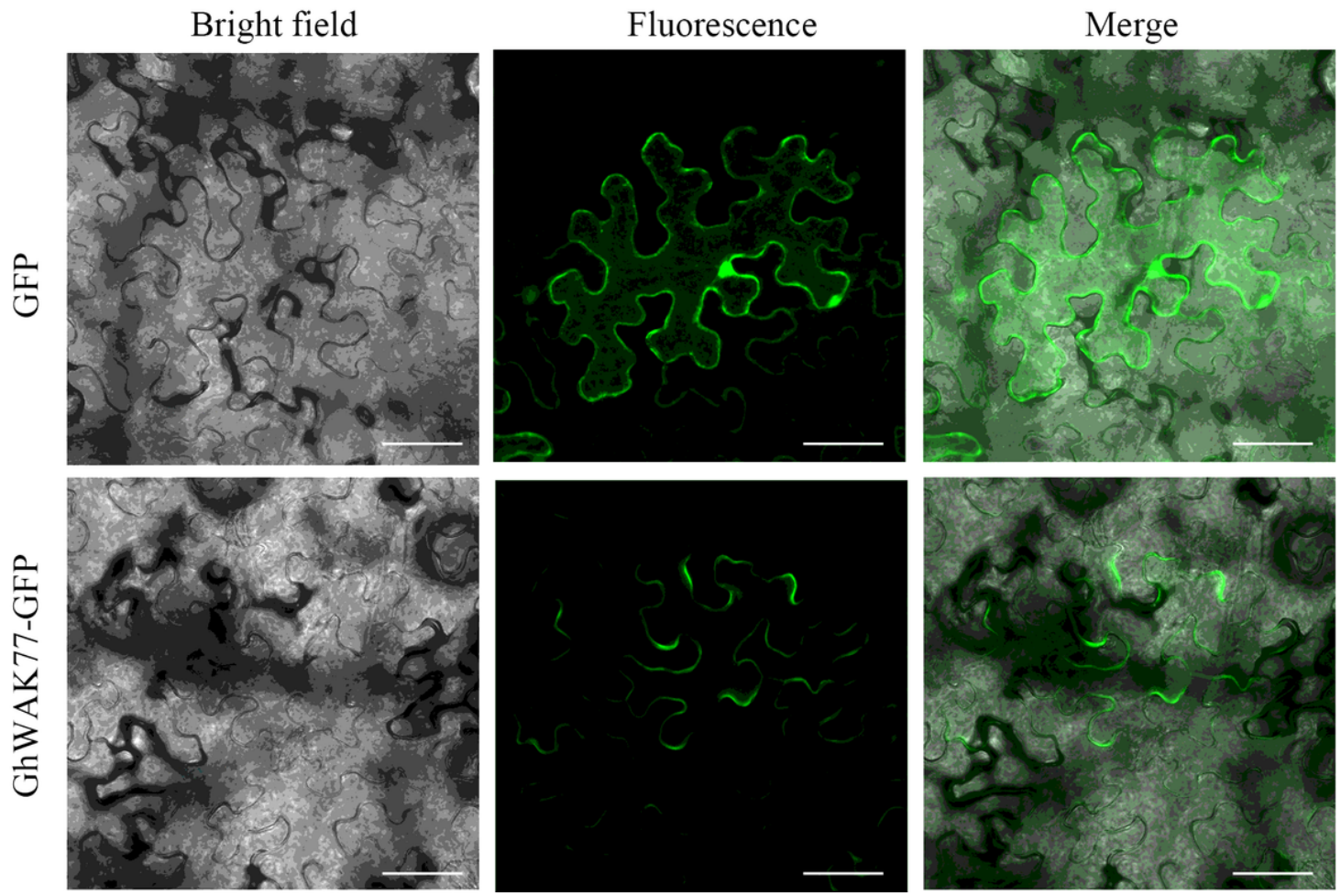

Figure 2

Subcellular localization of GhWAK77 in tobacco leaf epidermal cells. GFP (positive control) or GhWAK77 fused with the GFP (GhWAK77-GFP) proteins were transiently expressed in tobacco leaves via A. tumefaciens GV3101. At 48 h after agroinfiltration, GFP fluorescence was observed with confocal laser scanning microscope. Scale bars, 50 μm.

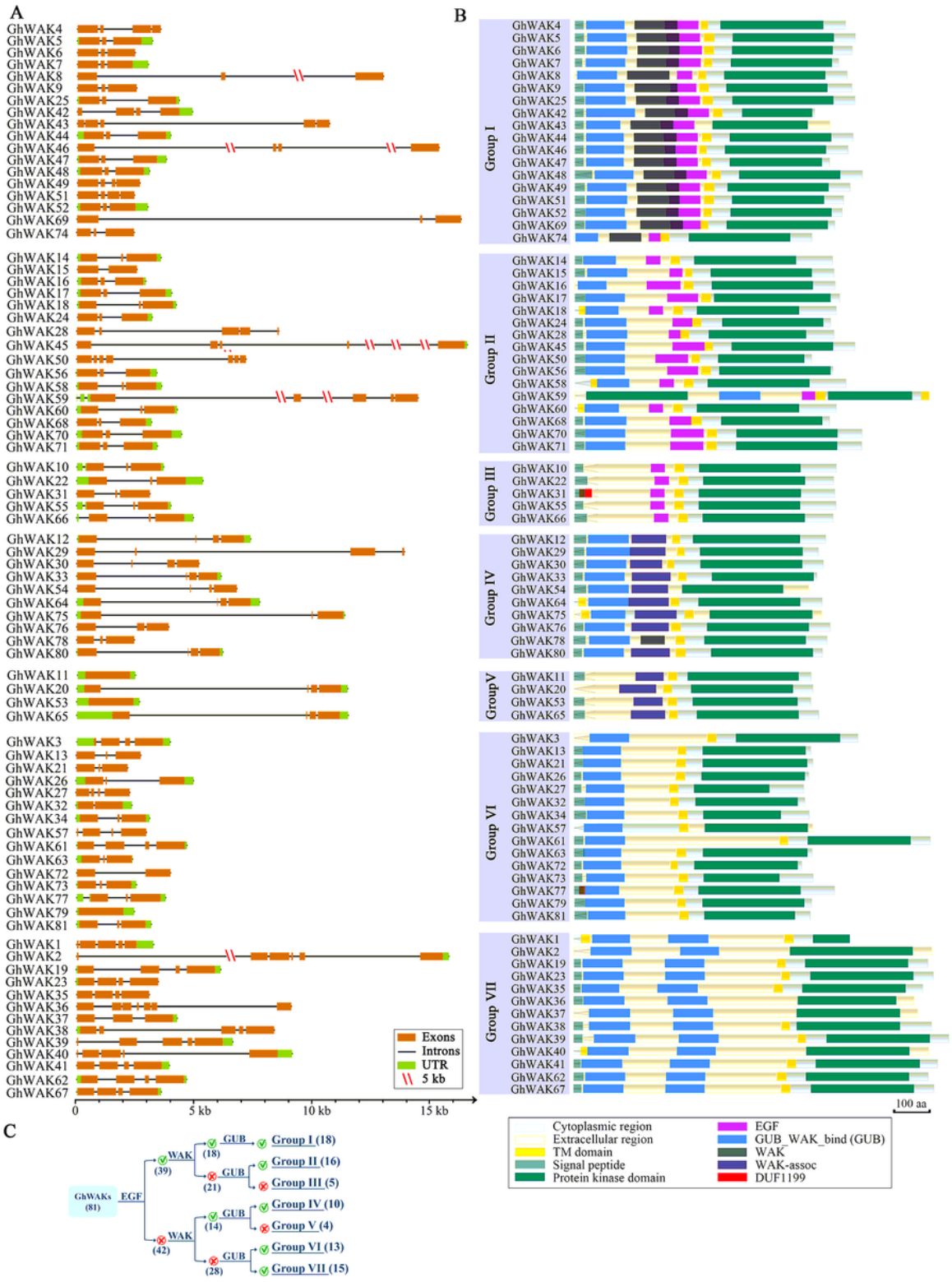

Figure 3

Gene structure and protein domain analysis of cotton WAK gene family. A, Gene structures of GhWAKs. B, Domain organization of GhWAKs. C, Grouping of GhWAKs. Based on the presence (green checkmarks) or absence (red crosses) of some domains, members of GhWAK family are divided into four groups. The numbers in brackets represent the number of GhWAKs.

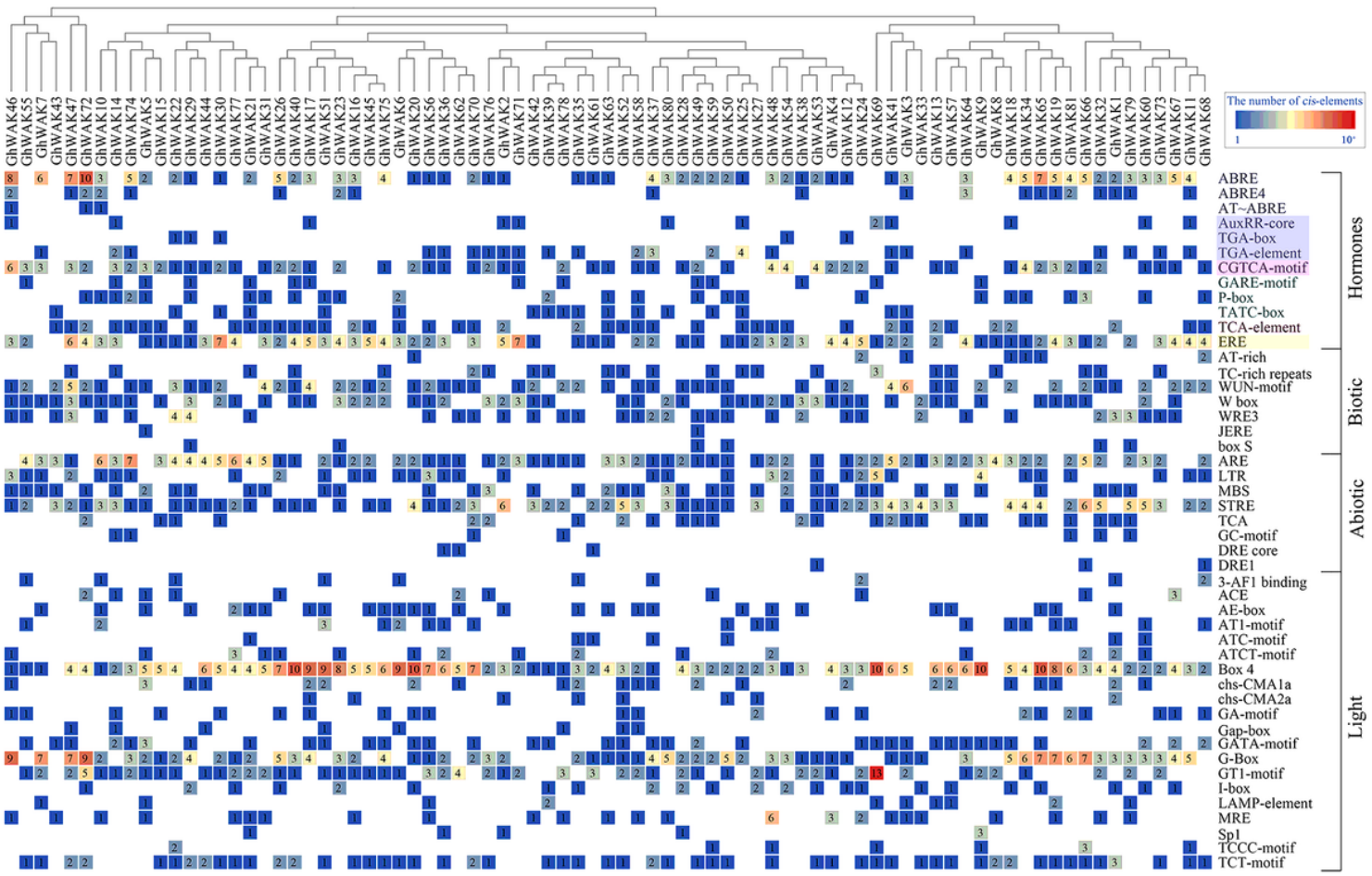

Figure 4

Potential cis-elements in a 2 kb 5' flanking region upstream from the start codon of each GhWAK. The number of each cis-element was shown, and the back-color changes from blue to red as the number increases. All the cis-regulatory elements were classified into four groups, including hormones, biotic, abiotic and light.

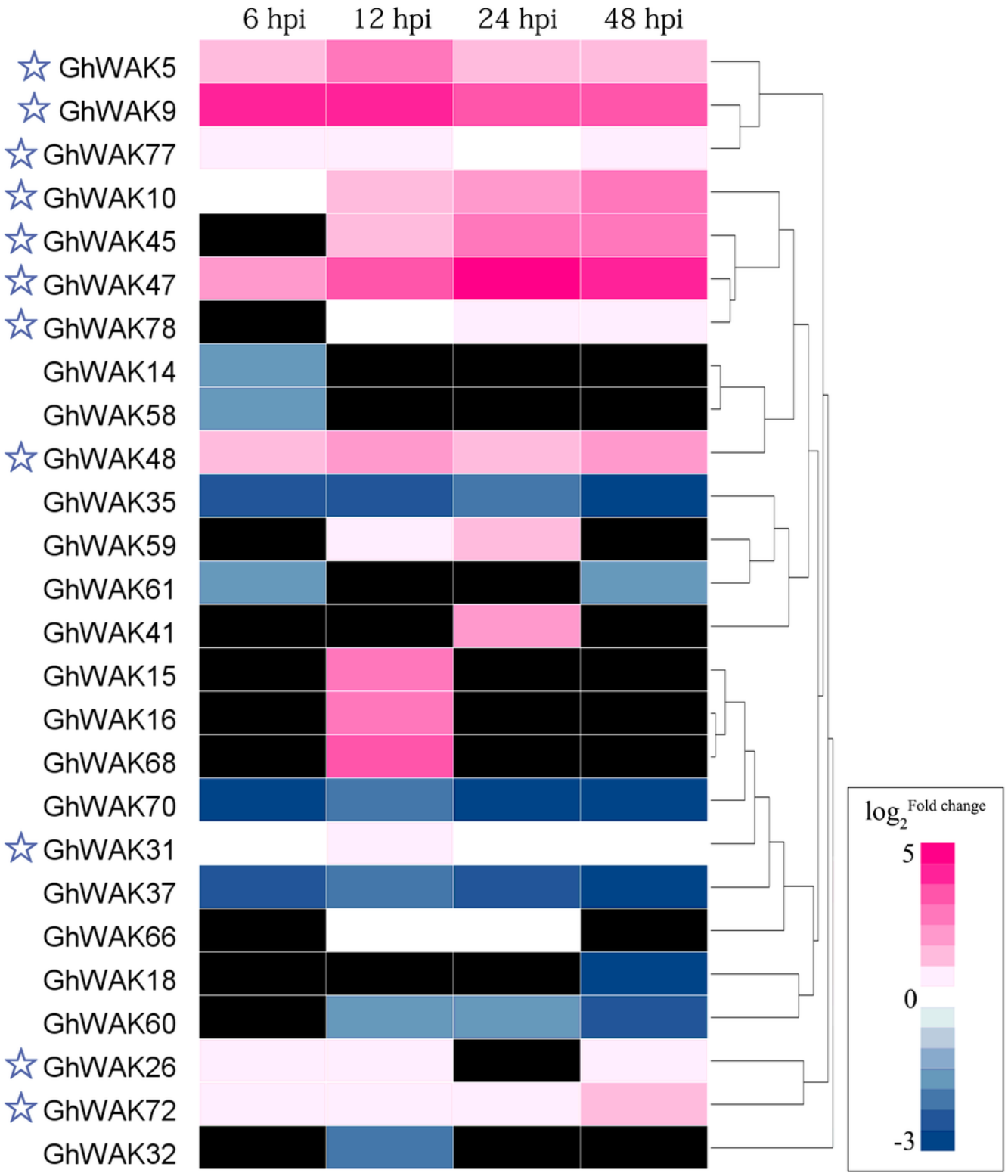

Figure 5

Heatmap representation for the expression patterns of 26 GhWAKs differentially expressed as a result of cotton inoculation with V. dahliae, compared to respective control. Expression levels of genes are shown as the Log2Fold change (FC). FC is the ratio of treatment FPKM to control FPKM values obtained from the RNA-Seq data. Higher and lower levels of transcription are indicated by pink and blue, respectively, and no detected expression is indicated by dark color.

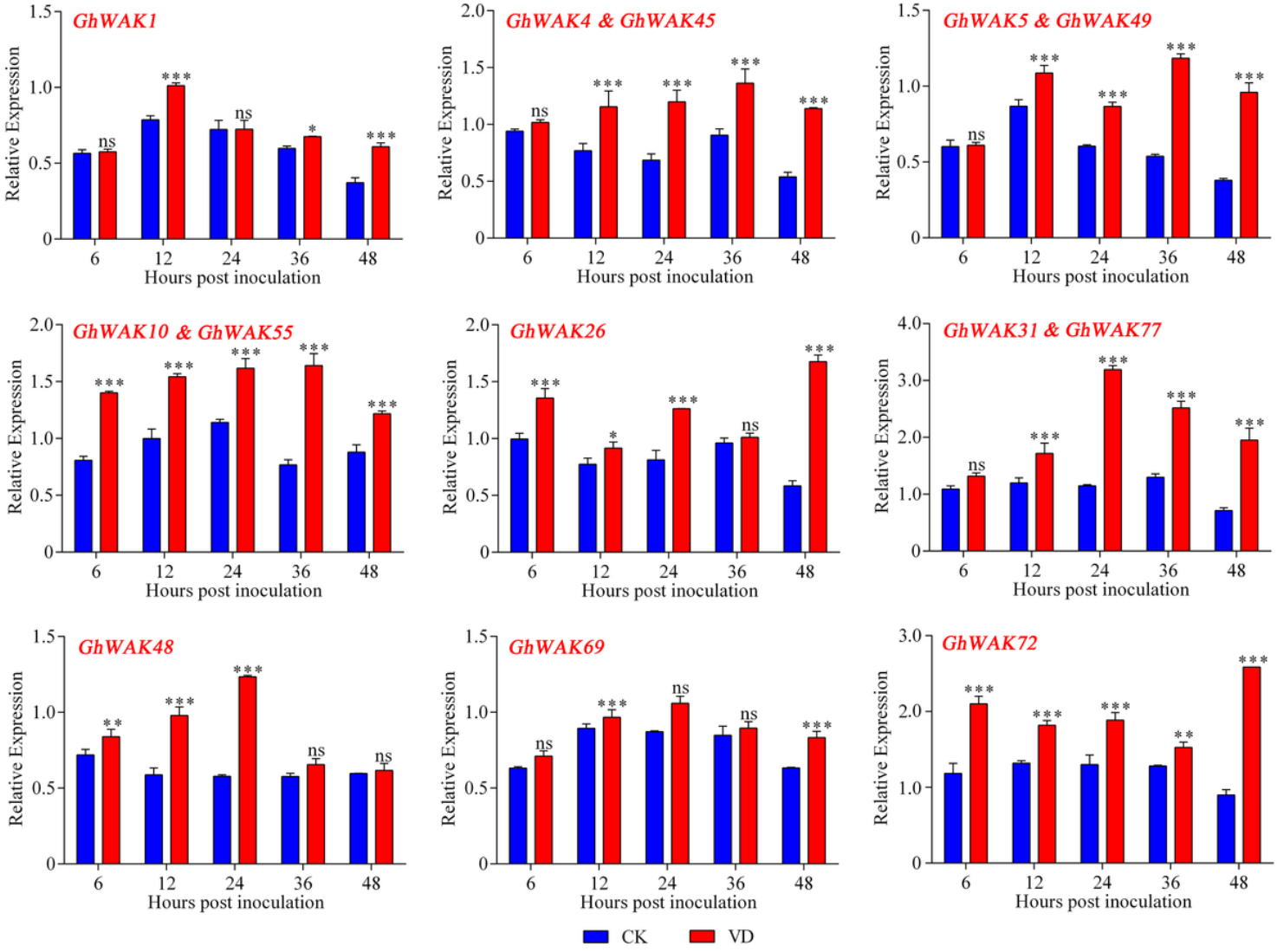

Figure 6

The expression analysis of GhWAKs from G. hirsutum ND601 induced with V. dahliae by qPCR. The relative gene expression level was calculated using the comparative 2-ΔΔCt method with GhHis3 as internal control. The error bars represent the standard deviation calculated from three independent experiments. CK, control; VD, V. dahliae.

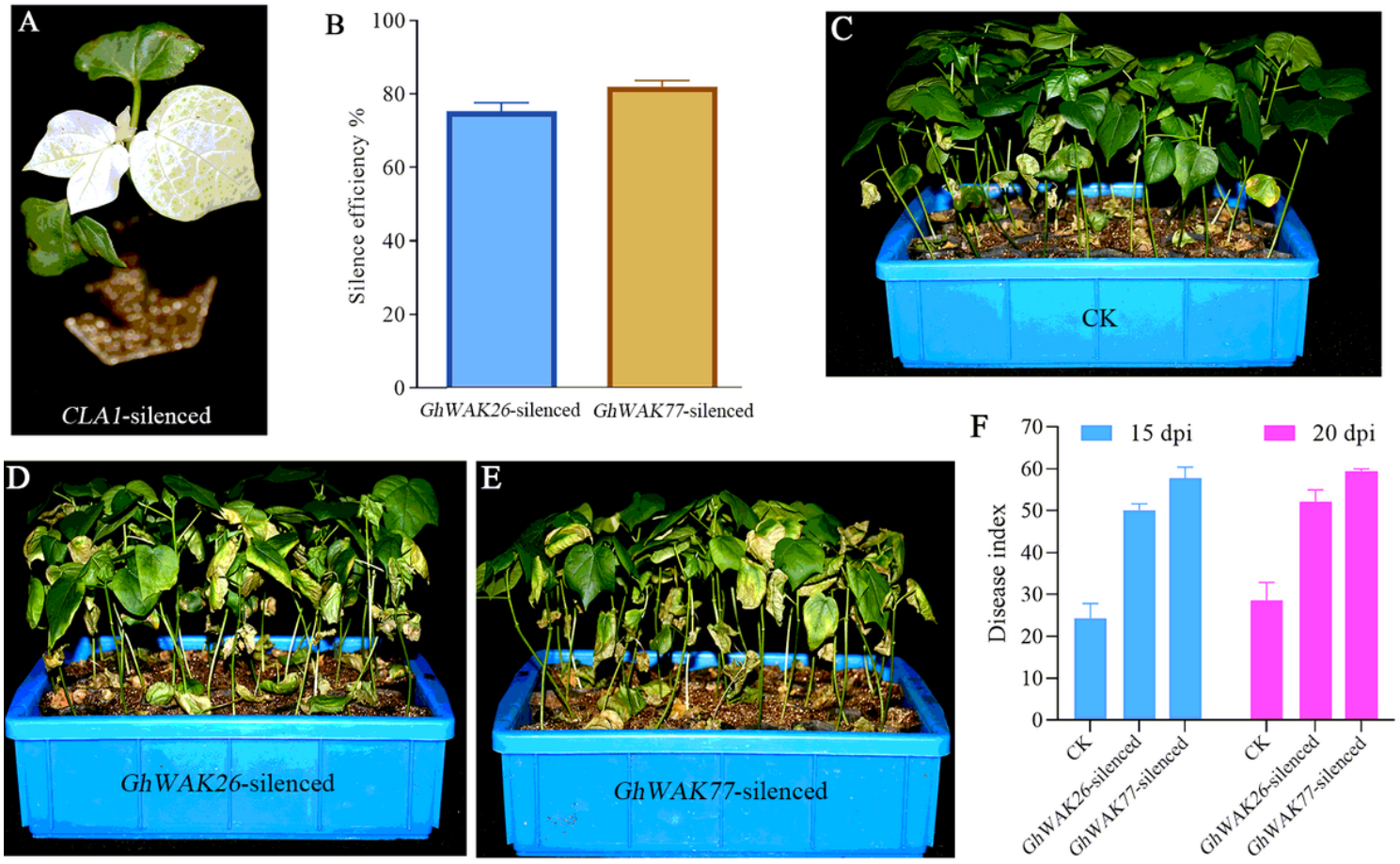

Figure 7

Silencing of GhWAKs in cotton compromises plant resistance to V. dahliae. A, Albinotic CLA1-silenced seedling served as the indicator of successful VIGS. B, VIGS reduces the expression of GhWAKs by about 80%. C, Disease symptoms on empty vector control after inoculation with V. dahliae at 15 dpi. D, Disease symptoms on GhWAK26-silenced plants after inoculation with V. dahliae at 15 dpi. E, Disease symptoms on GhWAK77-silenced plants after inoculation with V. dahliae at 15 dpi. F, The disease index of GhWAK26- and GhWAK77-silenced plants at 15 dpi and 20 dpi. The results were evaluated by three replications containing at least 30 plants each.

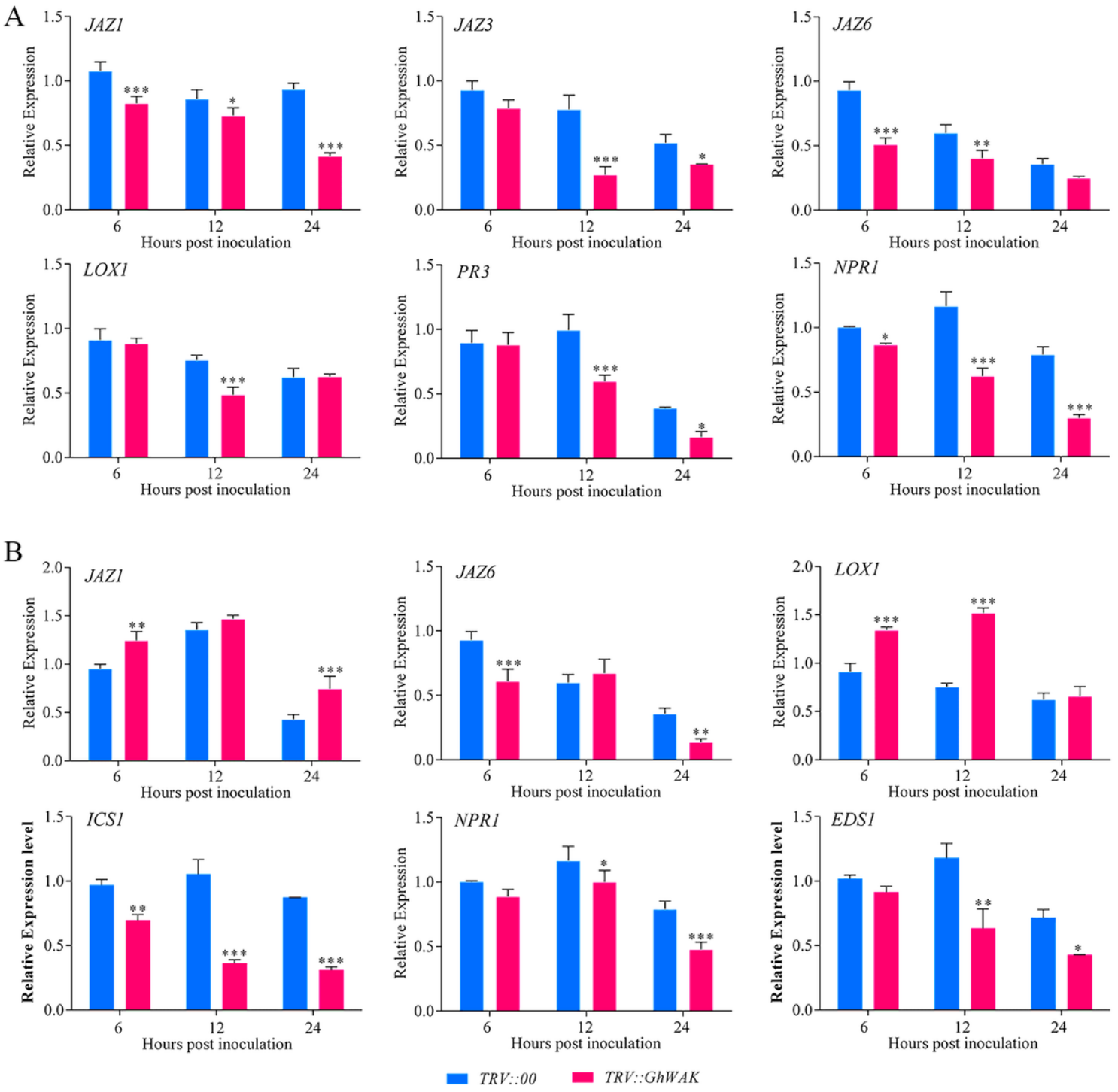

Figure 8

Silencing of GhWAKs effect on the expression of marker genes for JA and SA signaling pathways. A, The expression levels of six marker genes in GhWAK26-silenced plants upon inoculation with V. dahliae. B, The expression levels of six marker genes in GhWAK77-silenced plants upon inoculation with V. dahliae. JAZs, LOX1 and PR3 are the marker genes involved in JA signaling pathway. ICS1, NPR1 and EDS1 are the marker genes involved in SA signaling pathway. The results from three biological replicates are shown with mean ± SE. Asterisks represent P values (***P < 0.001, **P < 0.01, and *P < 0.05; Sidak's multiple comparisons test).

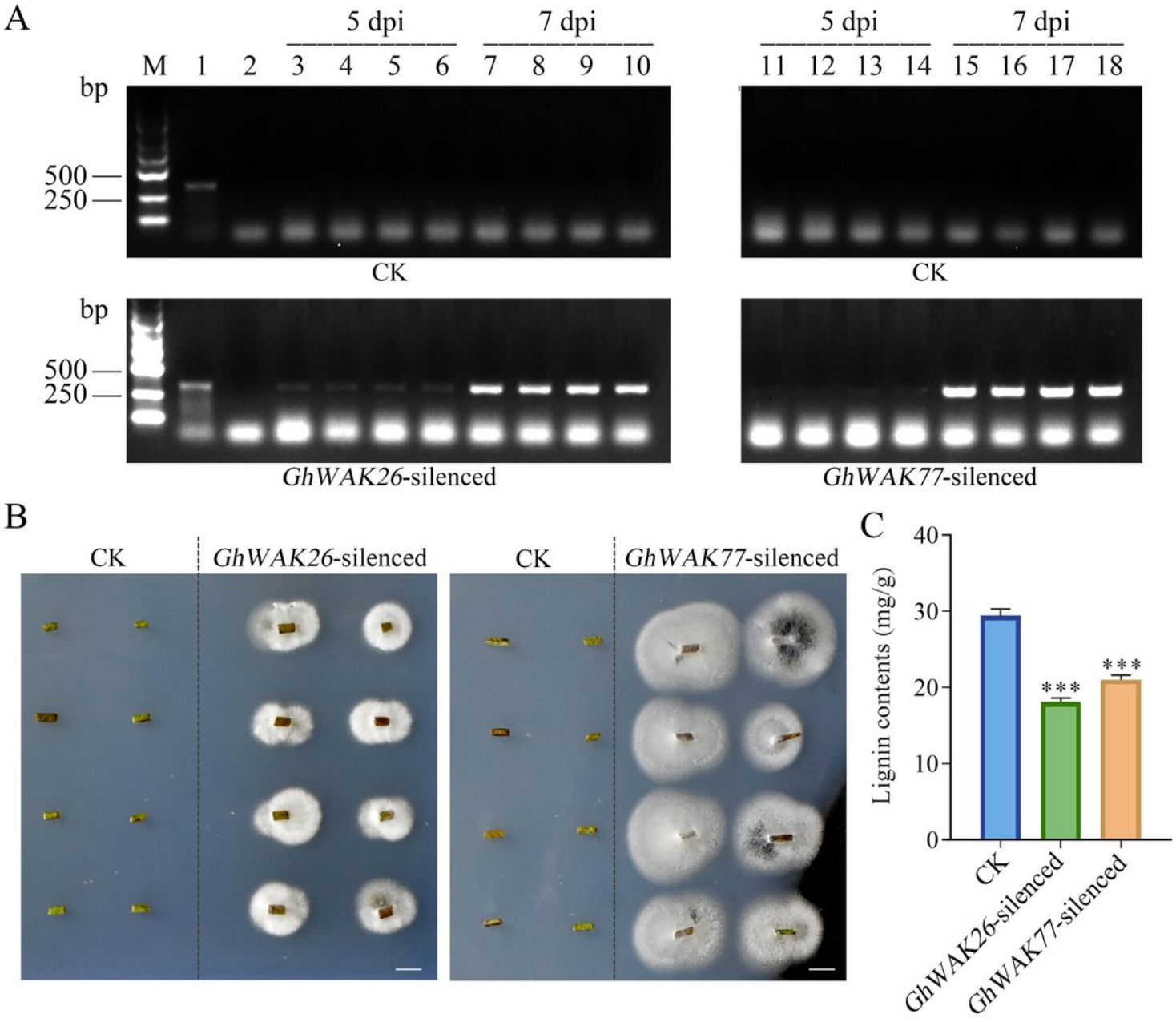

Figure 9

Silencing of GhWAKs increased the spread of V. dahliae in the cotton stems. A, Detection of V. dahliae in cotton stems at 5 dpi and 7 dpi by PCR. M, marker. PCR DNA templates were from V. dahliae spores as positive control (lane 1), water as negative control (lane 2), and cotton seedling stems (lane 3-18). B, Isolation of V. dahliae from the stems of GhWAK26-silenced and GhWAK77-silenced cotton plants by PDA cultivation. Bars = 0.5 cm. C, The lignin content in GhWAK-silenced plants and CK. The results from three biological replicates are shown with mean ± SE. Asterisks represent P values (***P < 0.001; Dunnett's multiple comparisons test).

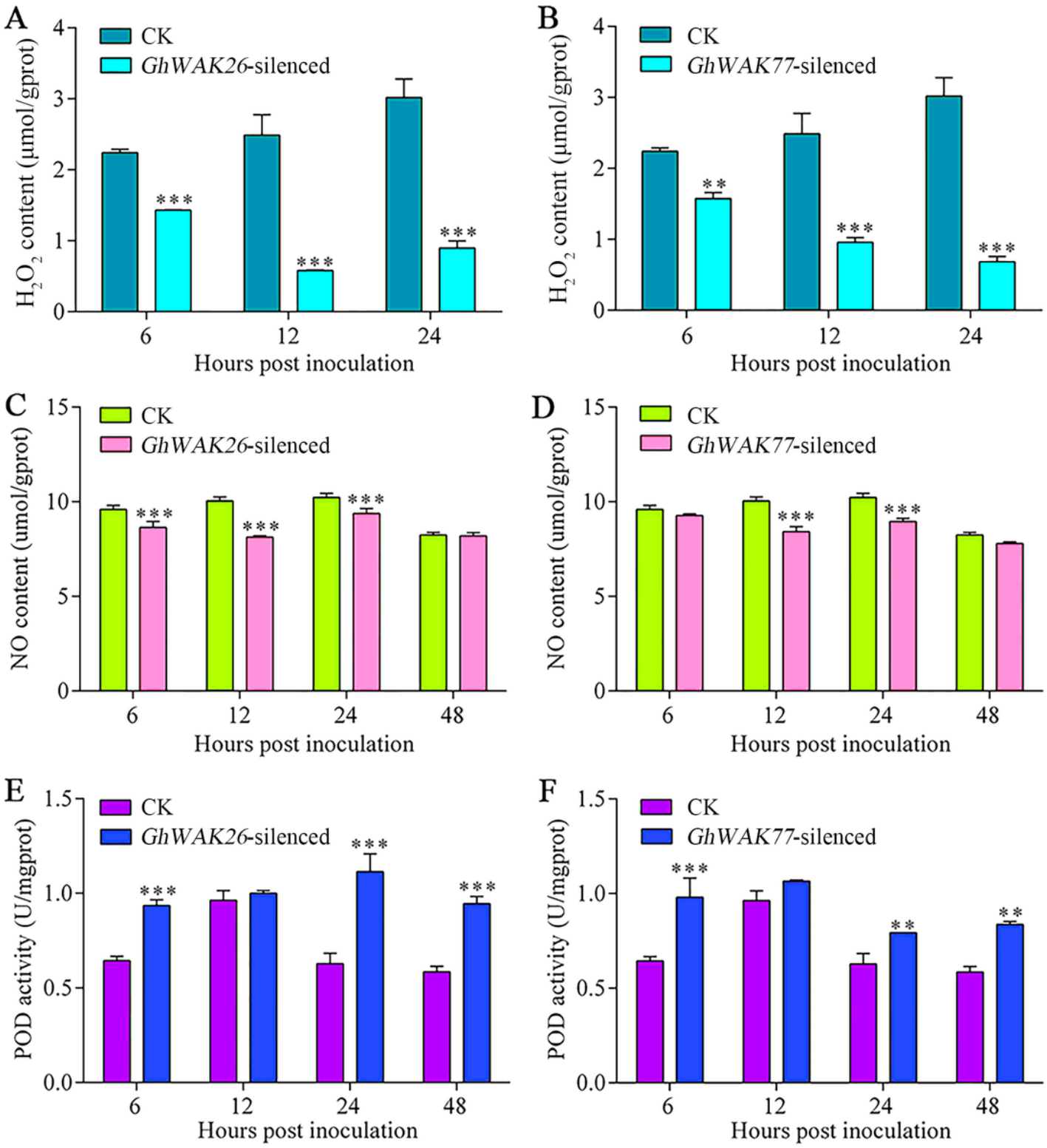

Figure 10

Silencing of GhWAKs dramatically compromised V. dahliae-induced accumulation of H2O2 (A and B) and NO (C and D), but enhanced POD activity (E and F). The results from three biological replicates are shown with mean ± SE. Asterisks represent P values (***P < 0.001, **P < 0.01; Sidak's multiple comparisons test).

Supplementary Files

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- FigureS1.jpg
- TableS1Primer.docx