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Posted Date: 25 February 2025

doi: 10.20944/preprints202502.2038.v1

Keywords: Glycosyltransferases; *Verticillium wilt*; *Gossypium hirsutum*; GhGT61



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Article

The Analysis of the Glycosyltransferase Activity Gene Family in *Gossypium hirsutum* and Functional Verification of GTs Conferring Resistance to Verticillium Wilt

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Abstract: Glycosyltransferases (GTs) play an important role in plant growth and development, and responses to biotic and abiotic stresses. However, the function of GTs gene family in cotton resistance to Verticillium wilt is limited. In the present study, transcriptome analysis revealed eight GTs upregulated in susceptible cotton varieties and downregulated in resistant cotton varieties during early *Verticillium dahliae* inoculation, indicating which were involved in regulating the infection of *V. dahliae* in cotton. Promoter analysis revealed a high prevalence of MeJA (methyl jasmonate) and ABA (abscisic acid)-related cis-acting elements among these GTs. Genome-wide and location analysis of the homologous genes showed that these GTs were relatively conserved in evolution. Furthermore, a Virus-Induced Gene Silencing (VIGS) experiment results demonstrated a reduction in disease resistance after *GhGT61* silencing. These insights not only deepen our understanding of the GT family's role in cotton but also provide a foundation for future research on the disease resistance mechanisms of these genes.

Keywords: glycosyltransferases; verticillium wilt; *Gossypium hirsutum*; *GhGT61*

1. Introduction

Cotton is a pivotal economic crop and the primary source of natural fiber for the textile industry [1]. However, it is severely affected by Verticillium wilt caused by *Verticillium dahliae*. In China, this disease affects over 50% of cotton fields annually, resulting in substantial economic losses [2]. *V. dahliae* is particularly difficult to control due to its persistence in soil as dormant microsclerotia [3]. Currently, there is no effective chemical control for cotton Verticillium wilt [4]. The most cost-effective and efficient strategy involves breeding and deployment of disease-resistant cotton varieties [5]. The scarcity of available resistance genes for genetic engineering has significantly constrained cotton molecular breeding. GTs can link sugar molecules to specific receptors and produce glycosidic bonds, which are present in most organisms and can be particularly important in plants.

Some natural glycosides or sugar esters with more stable structure were usually formed by glycosylation modification [6]. Glycosylation is crucial for promoting plant storage, intracellular transport, and regulating the balance of growth agents within plants [7]. One important role of GTs is responsible to catalyze plant glycosylation [8]. GTs transfer activated sugars to various plant molecules, including monosaccharides, oligosaccharides, polysaccharides, and non-carbohydrate substrates such as proteins, antibiotics, and lipids, leading to the glycosylation of these compounds [9]. GTs are essential for plant growth, development, and responses to biotic and abiotic stresses.

UGT73C7 is a glycosyltransferase that regulates disease resistance by redirecting the phenylpropanoid pathway and activating the TIR-type NLR protein SNC1 in *Arabidopsis thaliana* [10]. UGT76B1 can regulate the basic defense and SAR by coordinating the glycosylation of three defense activators SA, ILA, and NHP [11]. CsUGT87E7 encodes a SA carboxyl-glucosyltransferase and enhances the disease resistance of tea by regulating SA homeostasis [12]. *TaUGT6* is a novel UDP-glycosyltransferase gene, which can enhance the resistance of wheat to Fusarium head blight by converting deoxynivalenol (DON) into DON-3-glucoside [13]. However, few reports have reported on the role of *GTs* in cotton against *V. dahliae*.

In our previous study, we found that *GTs* are involved in the regulation of cotton's defense response to *V. dahliae*. In order to clarify the molecular function of *GTs* in cotton, the *GTs* were analyzed in *G. arboreum*, *G. raimondii*, *G. hirsutum*, and *G. barbadense* genomes, respectively. And the characteristics of *GTs* were comprehensively analyzed in *G. hirsutum*, including their chromosomal locations, motif distributions, gene structures, and cis-elements in the promoter regions. Additionally, the impact of *GhGT61* on *V. dahliae* was verified by VIGS. This study will preliminarily clarify the function of *GTs* and provide gene resources for molecular breeding of upland cotton resistant to Verticillium wilt.

2. Results

2.1. *GTs* Are Involved in the Response of Cotton to *V. dahliae* Infection

In order to screen the key resistance- associated genes of upland cotton responding to *V. dahliae* infection, we analyzed the differentially expressed genes (DEGs) in both resistant and susceptible upland cotton varieties after inoculation with *V. dahliae*. GO enrichment analysis revealed that the transferase activity and transferring glycosyl groups pathway were significantly enriched in both cotton varieties at 12 h post-inoculation. However, the expression patterns of these DEGs were completely opposite because of that the related DEGs were up-regulated and down-regulated in susceptible and resistant upland cotton, respectively (Figure 1A, B).

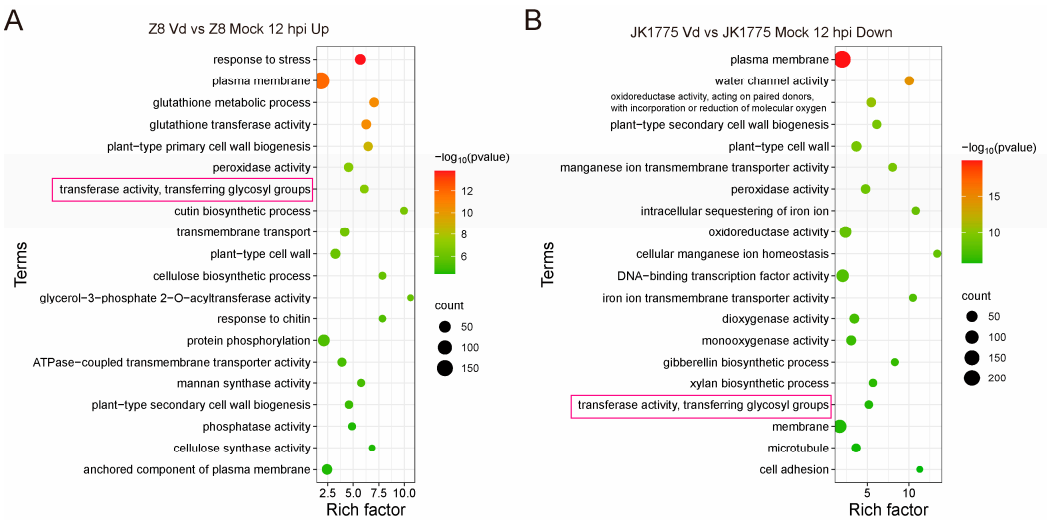


Figure 1. GO analysis of DEGs in response to *V. dahliae* infection at 12 hpi in resistant and susceptible upland cotton. A. GO analysis of up-regulating DEGs in response to *V. dahliae* infection at 12 hpi in susceptible upland cotton Z8. B. GO analysis of down-regulating DEGs in response to *V. dahliae* infection at 12 hpi in resistant upland cotton JK1775.

There were eight identical *GTs* involved in the transferase activity and transferring glycosyl groups pathway in both resistant and susceptible upland cotton (Figure 2A, B). These *GTs* may play a crucial role in the immune response of upland cotton to *V. dahliae*. In order to verify the reliability

of the selected *GTs* data, the differential expression levels of *GTs* were analyzed by qRT-PCR in the early stage of *V. dahliae* infection in both resistant and susceptible upland cotton. The results indicated that the expression level of the *GTs* were basically consistent with the results of transcriptome data (Figure 2 C). Notably, *GH_A04G1083* exhibited high expression in both inoculated and uninoculated upland cotton, with expression levels higher than those of other *GTs* (Figure2A, B, C). These results suggested that *GH_A04G1083* may play a role not only in the immune response to *V. dahliae* but also in other vital activities of upland cotton.

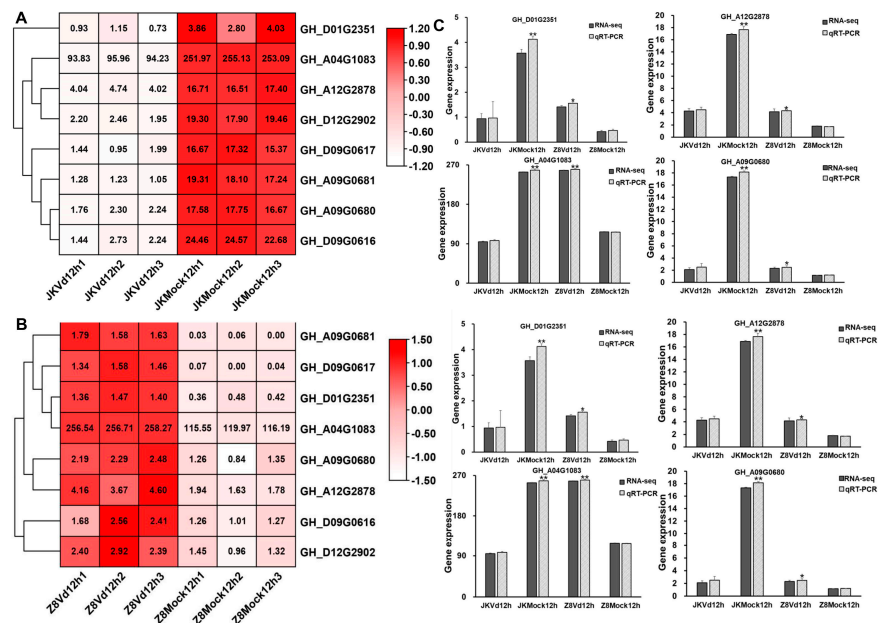


Figure 2. Expression analysis of eight *GTs* in response to *V. dahliae* infection in resistant and susceptible upland cotton at 12 hpi. A. Heat map of eight *GTs* in resistant upland cotton JK1775 in response to *V. dahliae* infection at 12 hpi. B. Heat map of eight *GTs* in susceptible upland cotton Z8 in response to *V. dahliae* infection at 12 hpi. C. qRT-PCR verification of *GTs* transcriptome data. Significance was determined using Duncan's multiple range test, indicated by * $p \leq 0.05$ and ** $p \leq 0.01$.

2.2. Protein Structure and Genetic Relationship of the Eight *GTs* in Cotton

To elucidate the biological function of the eight *GTs* furtherly, we analyzed their protein structure and genetic relationship. The results revealed that although the distribution of CDS sequences on the eight *GTs* showed certain differences (Figure 3C), *GH_A09G0681* and *GH_D09G0617*, *GH_A12G2878* and *GH_D12G2902*, *GH_A09G0680* and *GH_D09G0616* were clustered together, respectively, indicating a relatively close relationship (Figure 3A). And this six *GTs* were identified as members of GT family 61 based on their domain analysis (Figure 3B). Additionally, the *GH_D01G2351* and *GH_A04G1083* were classified under GT family 1 and GT family 2, respectively (Figure 3A).

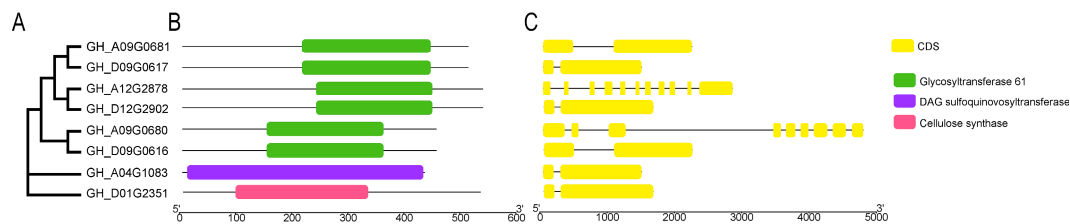


Figure 3. Phylogenetic tree, gene structure and conservative motif analysis of eight *GTs*. A. neighbor-joining phylogenetic tree of eight *GTs*. B. Prediction of conserved motifs in the *GTs*, with different colors representing

Group III

Group II

Group I

G. hirsutum

A. barbadense

A. arboreum

A. raimondii

Arabidopsis

Figure 4. Phylogenetic analysis of the GTs proteins from *G. hirsutum*, *G. barbadense*, *G. arboreum*, *G. raimondii*, and *A. thaliana*. The GTs proteins from the five species are indicated by different colors. The phylogenetic tree was constructed using MEGA X with the maximum likelihood method and 1000 bootstrap replications.

Promoter regions are crucial for determining gene expression patterns and functions. In order to predict the roles of the *GTs*, we analyzed the upstream cis-acting elements within their promoter regions. Interestingly, many of these elements were associated with plant hormones, including abscisic acid (ABA), gibberellin (GA), salicylic acid (SA), and MeJA, as well as abiotic stress factors such as anaerobic conditions, defense and stress responses, light responses, and low-temperature responses (Figure 5A). Among these elements, hormone-related elements (SA, MeJA, auxin, ABA, and gibberellin) account for a significant proportion, representing 50.93% of the total elements (Figure 5B). More importantly, defense and stress responses elements accounted for 5.66% of the total (Figure 5B). Notably, each *GT* contains at least one hormone-related cis-acting element, particularly

those related to ABA. Among the eight *GTs*, *GH_A09G0680*, *GH_D09G0616*, and *GH_D12G2902* lack MeJA-related elements. Collectively, these results indicate that *GTs* are crucial for conferring resistance to upland cotton against adverse environmental conditions.

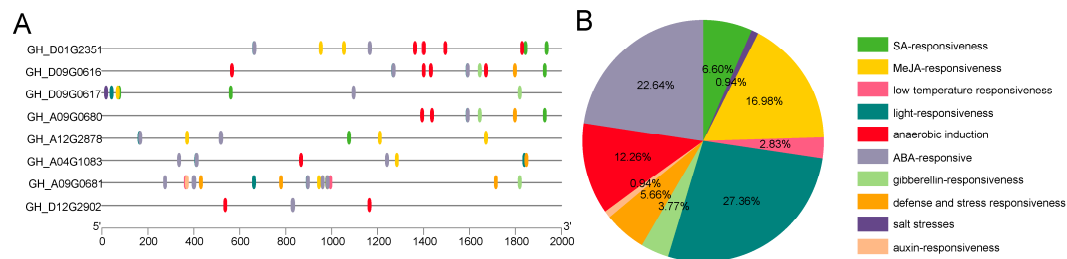


Figure 5. Cis-acting elements of *GTs* gene in upland cotton. A. Predicted cis-elements in the promoter regions of *GTs*. B. Proportion of different types of cis-elements. Ten groups of cis-acting elements were identified and represented by color-coded boxes.

2.4. Chromosomal Localization of Cotton GTs

To investigate the genomic distribution of cotton *GTs*, a chromosome map was constructed for 41 *GTs* in upland cotton. A total of 20 *GTs* were mapped to 9 chromosomes of the At subgenome, while 21 *GTs* were mapped to 10 chromosomes of the Dt subgenome (Figure 6). The distribution of *GTs* genes across At and Dt chromosomes is uneven, ranging from 1 to 6. Specifically, there are 6 *GTs* located on chromosomes A09 and D09, while 6 *GTs* located on chromosomes A02, A04, A06, A12, D02, D04, D06, D10, D12. Notably, the number and chromosomal location of certain *GTs* on the At subgenome are the same as those on the Dt subgenome, as evidenced by pairs such as A01-D01, A02-D02, A04-D04, A05-D05, A07-D07, A09-D09, and A12-D12.

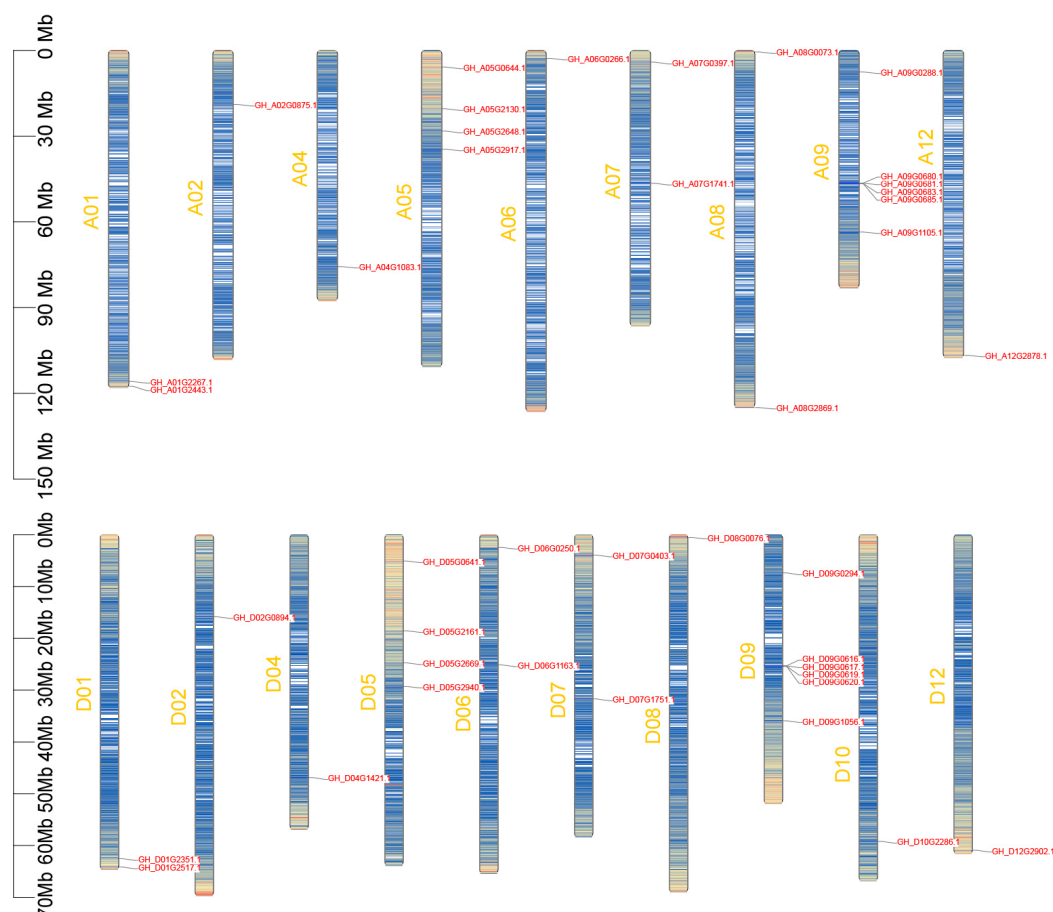


Figure 6. Chromosome distribution of GTs genes in *G. hirsutum*.

2.5. Synteny Analysis of GTs in Cotton

In order to investigate the fragment replication and tandem replication of GTs in upland cotton, the amplification mechanism of cotton GTs were analyzed using BLAST and MCScanX. The results suggested that six of the eight GTs formed gene clusters, including *GH_A04G1083-GH_D01G2517*, *GH_D01G2351-GH_A09G0685*, *GH_A12G2878-GH_A09G0680*, and *GH_D09G0616-GH_D12G2902*, which constituted four tandem clusters on chromosomes A12, A09, D09, and D12, respectively (Figure 7A). In contrast, *GH_D09G0617* and *GH_A09G0681* did not form gene clusters (Figure 7A). These four tandem duplications are inferred to have originated from polyploid ancestors, while *GH_D09G0617* and *GH_A09G0681* are believed to have arisen in *G. hirsutum* following its divergence from *G. barbadense*, as they are absent in *G. barbadense* (Figure 7B).

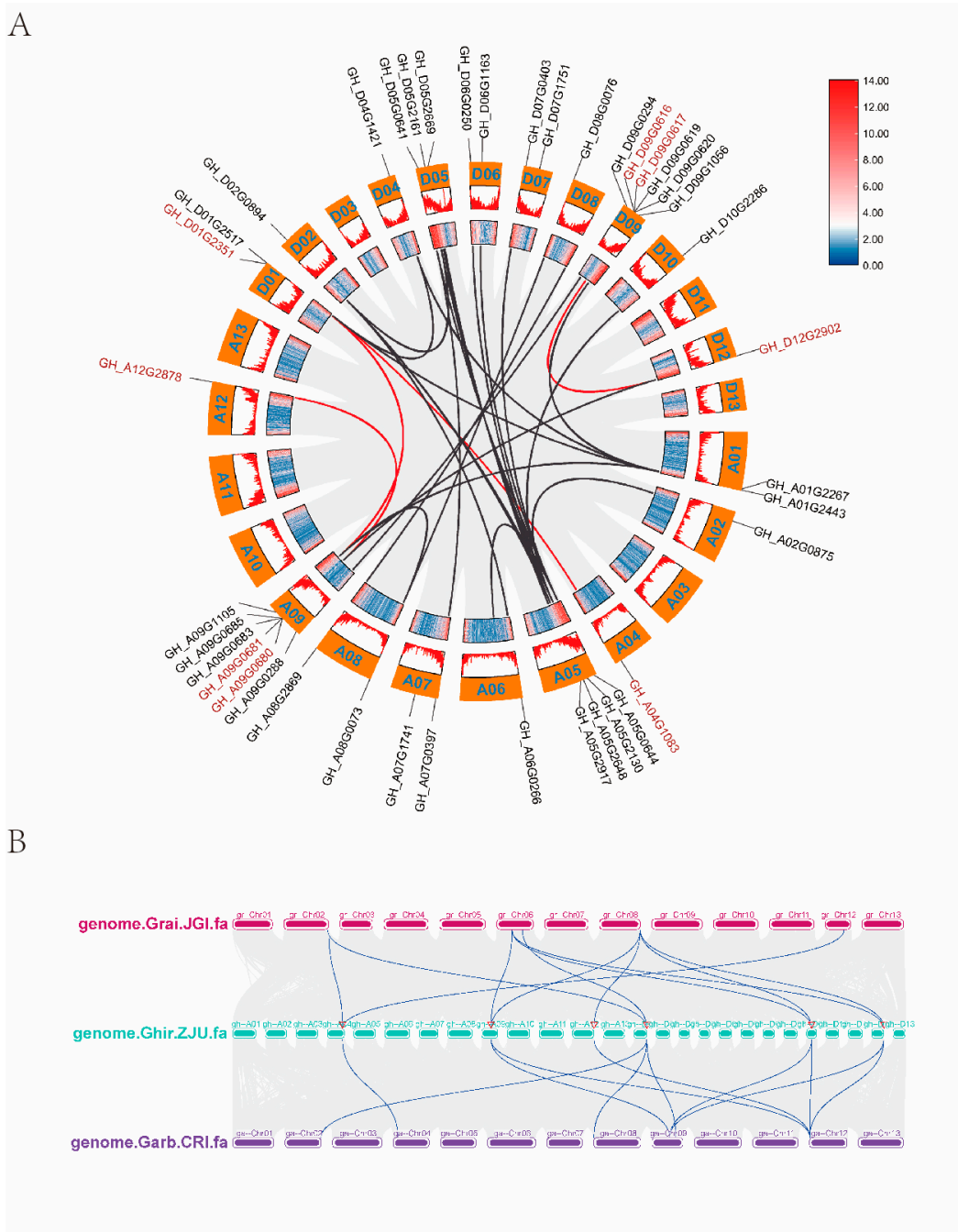


Figure 7. Intraspecific and interspecific collinearity analysis of eight GTs. A. Synteny analysis between At and Dt subgenomes in *G. hirsutum*. B. Synteny of repetitive gene pairs in three cotton species (*G. hirsutum*, *G. arboreum* and *G. raimondii*).

2.6. Silence of *GhGT61* *GhGTs* Affected the Disease Resistance of Cotton

To elucidate the role of *GhGTs* in cotton resistance to Verticillium wilt, VIGS and disease resistance assays were carried out. The results showed that an albino phenotype was observed at 14 dpi (Figure 7A). Following inoculation with *V. dahliae* 13 days after silencing, plants with suppressed *GhGT61* exhibited markedly reduced resistance to the *V. dahliae* (Figure 7B, F), with a significant reduction in expression level of *GhGT61* (Figure 7E). Histological analysis of longitudinal sections of cotton cotyledonary nodes revealed pronounced browning in TRV2:*GhGT61* plants compared to TRV2:00 controls (Figure 7C). Fungal biomass quantification via recovery culture showed significantly higher *V. dahliae* accumulation in stems of TRV2:*GhGT61* plants than in TRV2:00 controls (Figures 7D, G). These results collectively demonstrate that silencing *GhGT61* enhances cotton susceptibility to *V. dahliae*, underscoring its critical role in mediating resistance to Verticillium wilt.

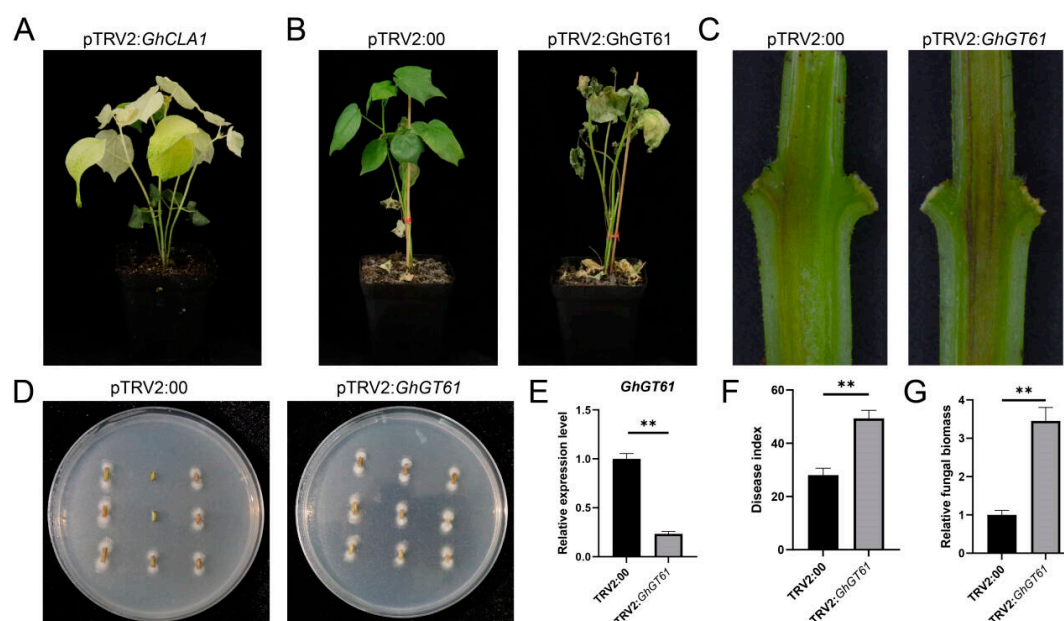


Figure 7. *GhGT61* Function in Cotton Resistance to Verticillium Wilt. A. VIGS-mediated silencing of *GhCLA1* resulted in an albino phenotype in cotton. B. Phenotype of silenced and normal plants inoculated with *V. dahliae* at 13 dpi. C. Vascular bundle browning observed in longitudinal stem sections of silenced and control plants. D. Fungal biomass recovery experiment in stems of silenced and control plants. E. The expression level of *GhGT61* in silenced and normal plants. F. Disease index of silenced and normal plants at 13 dpi. G. qRT-PCR quantification of *V. dahliae* biomass in stems of silenced and control plants. Significance was determined using Duncan's multiple range test, indicated by ** $p \leq 0.01$.

3. Discussion

In recent years, continuous cropping has resulted in a significant increase in Verticillium wilt incidence in the cotton industry, severely impacting cotton yields. The application of modern molecular techniques for identifying resistance-related genes has accelerated the breeding of disease-resistant varieties [14]. Exploring key genes associated with cotton's resistance to Verticillium wilt and elucidating their mechanism is crucial for developing new cotton germplasm resistant to Verticillium wilt [15]. Recent advancements in sequencing technology and decreasing costs have significantly improved and updated cotton genome sequencing efforts, providing a foundation for studying gene functions at the genomic level [16]. Transcriptome analysis can identify potential genes and molecular mechanisms involved in various biotic stress responses, playing a vital role in elucidating gene function [17]. A more profound comprehension of cotton genomics and genetics opens new pathways for investigating the *GTs* family.

Glycosylation is a common modification of plant secondary metabolites and plays various functions, including the regulation of hormone homeostasis, detoxification of exogenous substances, and biosynthesis and storage of secondary compounds. These processes are facilitated by specific subclasses of the GT family [18]. However, there are few studies on the biotic stress of cotton GTs. In the present study, we identified that there were eight GTs involved in cotton regulation of the defense response to *V. dahliae* in the early infection period. The GT family has remained conserved throughout the long-term evolution of cotton, consistent with its evolutionary relationships. Notably, the number of GTs in tetraploid cotton is approximately double that of diploid cotton species, further supporting the existence of two diploid ancestors for tetraploid cotton [19].

Gene function is closely linked to the differentiation of promoter region, which are the combination regions of many transcription factors (TFs) [20]. TFs usually regulate various aspects of plant growth, development, and stress resistance by regulating gene expression in responses to biotic and abiotic stress factors [21]. Analysis of the cis-elements in the promoter regions of these eight GTs revealed that the cis-elements with the strongest hormone response were ABA and MEJA response cis-elements. This suggests that these GTs may be involved in the response of cotton to *V. dahliae* through the ABA or MeJA pathway. Additionally, light-responsive cis-regulating elements account for a large proportion in the eight GTs with accounting for 27.336% of the total, indicating that GTs may participate in the regulation of light responses in cotton.

At present, the main goal of cotton breeding is to select high-yield and high-quality new varieties resistant to Verticillium wilt [22]. In this study, we analyzed the RNA-Seq data set (PRJNA1121084) of cotton inoculated with *V. dahliae* and found that eight GTs were differentially expressed in resistant and susceptible cotton after inoculation with *V. dahliae*, indicating that GTs may be involved in the defense process of cotton against *V. dahliae*. Subsequently, resistance of cotton to *V. dahliae* was weakened after silencing *GhGT61*. To deepen the understanding of GTs gene family, a lot of work is still needed to produce transgenic cotton and CRISPR/Cas9-mediated knockout in the future. It is necessary to verify the function, find important genes and analyze whether there is functional redundancy between GTs genes in the resistance to Verticillium wilt.

4. Materials and Methods

4.1. RNA-seq and qRT-PCR Analysis

The transcriptome data were obtained from the NCBI Sequence Read Archive database (Project ID: PRJNA1121084)[23]. Data were filtered and quality-controlled using Fastp software [24]. Clean data were aligned to the *G. hirsutum* TM-1 genome (ZJU-improved_v2.1_a1) [1] using HISAT2, and StringTie was used for read quantification. Gene expression was evaluated using the RPKM method [25]. For qRT-PCR, primers were designed using Primer 5.3 software based on the cDNA sequences of eight candidate genes. Expression levels were analyzed using root tissue cDNA as a template. Each sample was replicated 7 times, and *GhUBQ7* was used as an internal reference gene. qRT-PCR was performed as described previously [26].

4.2. Identification of GTs in Cotton

Genomic data of *G. arboreum*, *G. raimondii*, *G. hirsutum* and *G. barbadense*. were obtained from the CottonGen database (<http://www.cottongen.org/>). Homologous sequences were identified using BLAST with an e-value threshold of 1e-5. A total of 21 Ga and Gr, 41 Gh, and 40 Gb GTs were retrieved [27].

4.3. Analysis of Gene Structure and Conserved Motifs

Gene structure analysis was performed using TBtools to identify exons, introns, and untranslated regions of GTs in cotton. Domain analysis was performed using NCBI conserved domain database (CDD) to determine the domain types and positions. Subsequently, Gene exons, introns, and conserved motifs were visualized using Tbtools (v2.149).

4.4. Phylogenetic Analysis of GTs in Cotton

Multiple sequence alignments were performed using Clustal W in MEGA X (v11.0.13) with default settings (gap opening penalty = 10, gap separation distance = 4). A phylogenetic tree with 1000 bootstrap replicates was constructed using the neighbor-joining (NJ) method. The tree was visualized and edited using FigTree (<http://tree.bio.ed.ac.uk/software/Figtree/>).

4.5. Analysis of Cotton Glycosyltransferase Activity Gene Promoter Region

The 2,000 bp upstream DNA sequences of the GTs genes were extracted from the CottonFGD database (<http://www.cottonfgd.org/>). Cis-acting elements were predicted using the PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [29], and their positions were visualized using TBtools.

4.6. VIGS of GhGT61

RNA was isolated from cotton roots and reverse-transcribed into cDNA. A 300 bp CDS fragment of *GhGT61* (GH_D12G2902) was amplified using primers with BamHI and EcoRI restriction sites. The fragment was cloned into the TRV2 vector by homologous recombination and transformed into *Agrobacterium* GV3101. The transformed TRV2 vector was injected into Z8 with completely flattened cotyledons, and then the transformed seedlings were transferred to a growth chamber at 25°C with a light/dark photoperiod of 16 h/ 8 h. VIGS efficiency was assessed using the TRV2:*GhCLA* construct. After 15 d, the expression level of the target gene was detected, and the successfully silenced plants were infected with V592 [30].

5. Conclusions

This study identified eight GTs that were differentially expressed in cotton varieties susceptible and resistant to *Verticillium* wilt through RNA-Seq analysis. The analysis of gene number, chromosome location and evolution revealed that the GTs family is conserved in cotton. The presence of ABA- and MeJA-responsive cis-acting elements suggests that these GTs may participate in the response of cotton to *V. dahliae* infection through these hormonal pathways. Additionally, the presence of photoresponsive elements in the cis-acting elements of each gene suggests that GTs may be involved in the regulation of cotton photoperiod. VIGS experiments further revealed that *GhGT61* is involved in the resistance process of cotton to *Verticillium* wilt. This study provides a foundation for further research on the role of GTs in cotton and offers insights for breeding efforts aimed at developing more resistant cotton varieties.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: List of primers used in this study.

Author Contributions: Conceptualization, Y.Y and H.X.; methodology, M.Z.; software, M.Z; validation, M.Z, F.Z and G.S.; formal analysis, Y.M.; investigation, M.Z and X.Z; resources, Y.Y and H.X.; data curation, M.Z; writing—original draft preparation, M.Z; writing—review and editing, M.Z, X.Z, Y.Y, and H.X; visualization, H.X.; supervision, S.Z and H.X.; project administration, Y.Y and H.X.; funding acquisition, X.Z and H.X. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Shihezi University high-level talents research project(2022ZK015), Bingtuan Science and Technology Program (2022ZD056, 2023CB007-08), and the Earmarked fund for XJARS-Cotton (XJARS-03).

Data Availability Statement: The data used in this study were from the National Center for Biotechnology Information (NCBI), BioProject ID: PRJNA1121084.

Acknowledgments: We thank senior researcher Yu Yu from Xinjiang Academy of Agricultural Reclamation Sciences for his assistance with the materials used for experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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