BMC Plant Biology

RESEARCH Open Access

# Pan-genome analysis of GT64 gene family and expression response to Verticillium wilt in cotton

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## Abstract

**Background** The GT64 subfamily, belonging to the glycosyltransferase family, plays a critical function in plant adaptation to stress conditions and the modulation of plant growth, development, and organogenesis processes. However, a comprehensive identification and systematic analysis of GT64 in cotton are still lacking.

**Results** This study used bioinformatics techniques to conduct a detailed investigation on the GT64 gene family members of eight cotton species for the first time. A total of 39 *GT64* genes were detected, which could be classified into five subfamilies according to the phylogenetic tree. Among them, six genes were found in upland cotton. Furthermore, investigated the precise chromosomal positions of these genes and visually represented their gene structure details. Moreover, forecasted *cis*-regulatory elements in *GhGT64s* and ascertained the duplication type of the *GT64* in the eight cotton species. Evaluation of the Ka/Ks ratio for similar gene pairs among the eight cotton species provided insights into the selective pressures acting on these homologous genes. Additionally, analyzed the expression profiles of the GT64 gene family. Overexpressing *GhGT64_4* in tobacco improved its disease resistance. Subsequently, VIGS experiments conducted in cotton demonstrated reduced disease resistance upon silencing of the *GhGT64_4*, may indicate its involvement in affecting lignin and jasmonic acid biosynthesis pathways, thus impacting cotton resistance. Weighted Gene Co-expression Network Analysis (WGCNA) revealed an early immune response against *Verticillium dahliae* in *G. barbadense* compared to *G. hirsutum*. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis indicated that some *GT64* genes might play a role under various biotic and abiotic stress conditions.

**Conclusions** These discoveries enhance our knowledge of GT64 family members and lay the groundwork for future investigations into the disease resistance mechanisms of this gene in cotton.

**Keywords** Upland cotton, GT64, WGCNA, Expression pattern, VIGS, Transgenic tobacco

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## Background

*Glycosyltransferases* (GTs; EC 4.x.y) are essential metabolic enzymes in living organisms, catalyzing the transfer of sugar moieties to acceptor substrates and facilitating a wide range of biochemical reactions involved in carbohydrate metabolism. These reactions result in the production of glycosylated products such as polysaccharides, glycolipids, and glycoproteins. *Glycosyltransferases* are widely present in bacteria, fungi, plants, and animals, playing crucial biological roles [1]. They are involved in numerous physiological processes, including cell signaling, regulation of sugar metabolism, and modification of biomolecules. By regulating the degree of glycosylation of substrates, *glycosyltransferases* control the balance of sugar metabolism and energy production within cells. Additionally, *glycosyltransferases* participate in the virulence mechanisms of pathogenic microorganisms and immune responses [1]. Plants possess a diverse array of *glycosyltransferases* (GTs) for synthesizing various disaccharides, oligosaccharides, and polysaccharides, as well as unique secondary metabolites not found in other organisms. The Carbohydrate-Active Enzymes Database (CAZy) currently documents 114 GT families, encompassing more than 900,000 *GT* genes. Over 1000 potential *GT* genes were detected in the model plants *Arabidopsis* and rice [2, 3].

Most glycosyltransferases (GTs) can be categorized into three primary structural folds based on their conserved three-dimensional structures: GT-A, GT-B, and GT-C. The GT-A enzymes feature a topology comprising two closely linked β/α/β Rossmann-like domains, while GT-B enzymes possess two opposing Rossmann-like domains with a central cleft that acts as the catalytic center [4]. GT-A enzymes responsible for targeting the Golgi in most eukaryotes typically have a brief cytoplasmic tail, followed by a transmembrane domain, and connecting to an extended helical region that forms a compact catalytic domain within the Golgi lumen. In contrast, GT-C enzymes are proteins that are integrated into the membrane [5]. Within plants, the GT64 family adopts the GT-A structure and is characterized by an α-1,4-acetylglucosamine transferase domain. Members of this family are present in a diverse range of species, including animals, plants, fungi, and algae [6]. The first identified member of the GT64 family in plants is the *Arabidopsis* ectopic phloem cells 1 (*AtEPC1*) gene, which plays a role in cell division and elongation processes during wood formation. Remarkably, the epct1 mutant also exhibits significant ectopic chitin deposition in certain specific tissues, similar to the situation observed when leaves are infected with powdery mildew [7, 8]. Another *Arabidopsis* GT64 family member, *At5g04500*, has been identified as glucosamine inositol phosphorylceramide transferase 1 (*GINT1*), responsible for adding UDP-GlcNAc to GIPC. Overexpression of *OsGT64* down-regulated the expression of several defense-associated and cell wall synthesis-associated genes, and enhanced the sensitivity to rice blast [9, 10]. Apart from these findings, there are limited reports on *GT64* genes in plants.

Cotton verticillium wilt is a disease caused by fungi, known as the "cancer" of cotton, which seriously affects the yield and quality of cotton. Cotton is an important fiber crop and serves as an ideal model for studying polyploidy and species evolution [11]. Cotton is often affected by various abiotic and biological stress factors throughout its developmental cycle [12–14]. Harsh external environments can adversely affect cotton growth, leading to reduced yield and fiber quality. Therefore, enhancing plant stress tolerance can improve plant adaptability to stressful conditions, with gene engineering being a crucial technological approach [15–17]. The genus *Gossypium* comprises 45 diploid species and seven tetraploid species [18–21]. The development of cotton genome sequencing and assembly has established a basis for researching cotton gene families [22, 23].

In this investigation, utilizing prior transcriptome sequencing findings, we used the analysis method of WGCNA to screen for some hub genes related to disease resistance. Through qRT-PCR, we identified *GH_D04G0699 (GhGT64_4)* as a candidate gene for resistance to Verticillium wilt in upland cotton [24]. Subsequently, we conducted a comprehensive identification of GT64 gene family members in eight cotton species. Bioinformatics analysis and expression pattern analysis were also performed. In addition, we also verified the effect of *GhGT64_4* on tobacco and cotton.Through heterologous overexpression in tobacco and virus-induced gene silencing (VIGS) experiments, it confirmed the involvement of *GhGT64_4* in the process of resistance to Verticillium wilt in cotton.This study laid a foundation for studying the molecular mechanism of resistance of upland cotton to Verticillium wilt.

## Results

### Identification of GT64 family

Following a comparative search using the local BLASTP program, candidate sequences were identified and their conservation domains confirmed through Pfam, SMART, and CDD analysis. Subsequently, a total of 39 GT64 genes were identified across eight cotton species, with three genes in each of *G. herbaceum* (A1), *G. arboreum* (A2), and *G. raimondii* (D5), and six genes in *G. hirsutum* (AD1), *G. mustelinum* (AD4), *G. barbadense* (AD2), *G. tomentosum* (AD3), and *G. darwinii* (AD5). Notably, the number of genes in the D subgenome *G. raimondii* (D5) and the A subgenomes *G. herbaceum* (A1) and *G. arboreum* (A2) are all three, while the count of GT64 family members in the five tetraploid cotton species is twice that

of the three diploid cotton species, with each having six genes. These genes were subsequently renamed based on their chromosomal locations(Table S1).

The physicochemical properties of the GT64 gene family members in the eight cotton species were then assessed. The GT64 amino acid length ranged from 329 to 783 residues, with an average of 487 residues. The range of molecular weights for the proteins were 37.74 to 88.81 kDa, with an average of 55.22 kDa, while the isoelectric point (pI) varied from 8.31 to 9.4, averaging 8.92(Table S1).

### Construction of the phylogenetic tree of GT64 gene family members

A systematic phylogenetic analysis was conducted to explore the evolutionary relationships within the GT64 gene family across eight cotton species (Fig. 1). The study included the creation of phylogenetic trees of 39 GT64 protein sequences.The GT64 proteins were categorized into five subfamilies labeled as Class1 through Class5. Class1 exhibited the highest membership with thirteen members, followed by Class2 with nine members, and the fewest members belonged to Class5, totaling four. In Class1, each of the five tetraploid cotton species contained two members, while each of the three diploid cotton species had one member. Notably, *G. herbaceum* (A1) was absent in Class2. In Class3, all cotton species, except *G. herbaceum* (A1) and *G. arboreum*, were represented by one member each. *G. raimondii* was not present in Class4. Within Class5, *G. herbaceum* (A1), *G. mustelinum* (AD4), *G. tomentosum* (AD3), and *G. darwinii* (AD5) were the only species with one member each. Of particular interest is that in phylogenetic analysis, two diploid *Gossypium* species and two tetraploid *Gossypium* species often cluster together, indicating that upland cotton and island cotton have originated from two diploid *Gossypium* species [20].

### Chromosomal location of *GT64* genes in eight cotton species

We conducted a visual analysis of 39 *GT64* genes in the cotton genome. For instance, in *G. hirsutum* (Fig. 2C), six genes were identified across chromosomes A05, A12, D04, D05, and D12, with an equal representation of genes

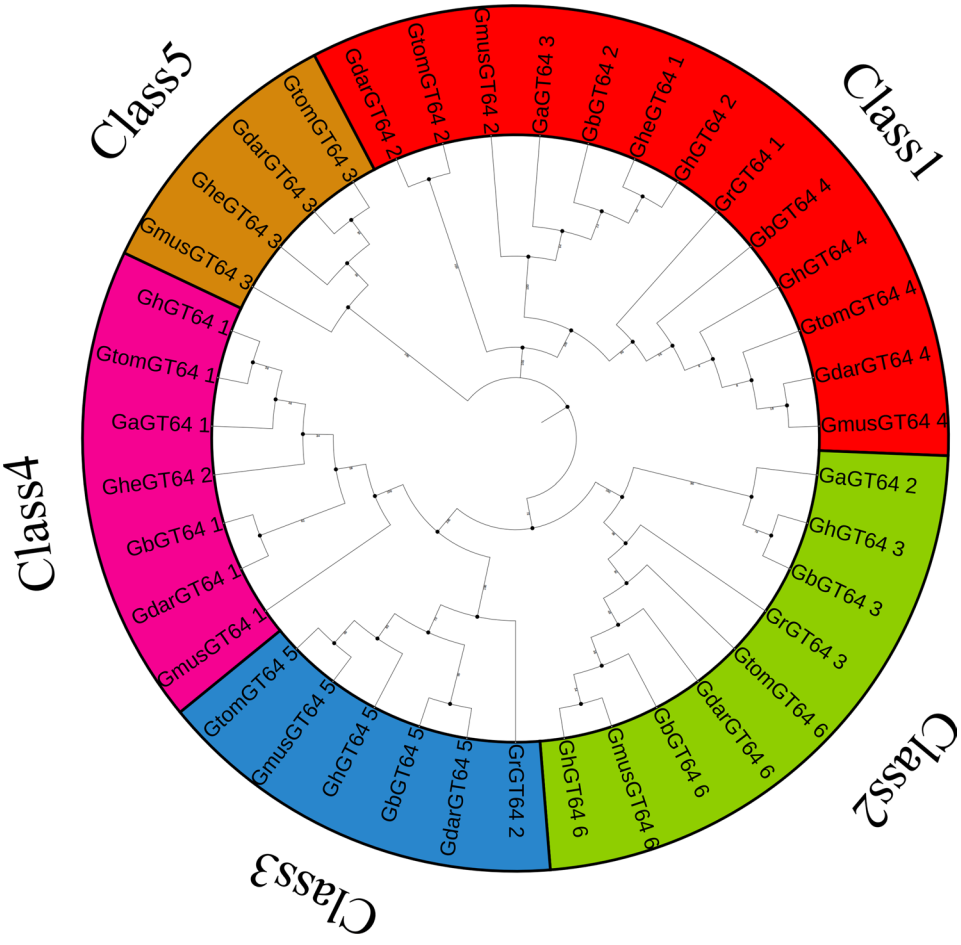


**Fig. 1** A phylogenetic analysis of the GT64 family members in eight species of the cotton species

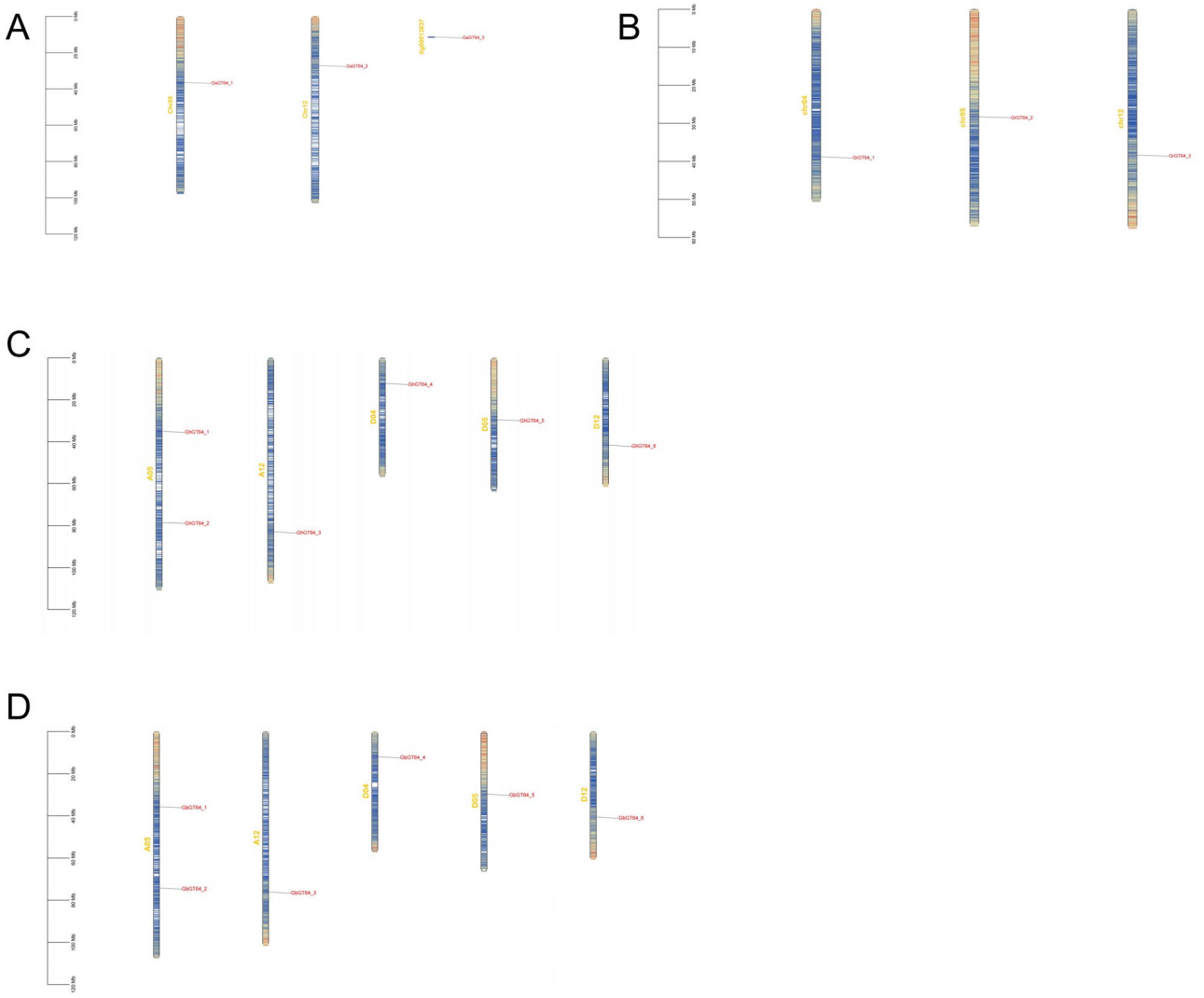


**Fig. 2** The physical locations of the *GT64* genes on the chromosomes, (**A**)-(**D**) represent *G. arboreum*, *G. raimondii*, *G. hirsutum*, *G. barbadense*

in both the A and D subgenomes. Notably, chromosome A05 harbored two genes, while the remaining chromosomes each contained one gene. The distribution pattern of *GT64* genes in *G. barbadense* mirrored that of *G. hirsutum* (Fig. 2D). In *G. arboreum* (Fig. 2A), three *GT64* genes were located on chromosomes Chr05 and Chr12. Similarly, in *G. raimondii*, three *GT64* genes were present on chromosomes Chr04, Chr05, and Chr12 (Fig. 2B), with the distinct occurrence of one *GT64* genes solely on chromosome Chr04, setting it apart from *G. arboreum*.

In *G. herbaceum* (Fig. 3A), similar to *G. raimondii*, the *GT64* genes were distributed on chromosomes Chr04, Chr05, and Chr12. In the remaining cotton species, tetraploid species *G. darwinii*, *G. mustelinum*, *G. tomentosum*, and *G. mustelinum* exhibited a distribution pattern consistent with *G. hirsutum* and *G. barbadense*, with all six *GT64* genes located on chromosomes A05, A12, D04, D05, and D12 (Fig. 3B-D).

### Basic analysis of *GhGT64s*

We conducted phylogenetic tree construction and analysis of motifs and gene structures of six genes in upland cotton (Fig. 4). The results indicated that among the six members in *G. hirsutum*, a total of 10 motifs were identified. Specifically, *GhGT64_1* and *GhGT64_5* encompassed all 10 motifs, *GhGT64_2* and *GhGT64_4* contained six motifs each (motif1-motif4, motif6, and motif7), while *GhGT64_3* and *GhGT64_6* contained motifs 1 to 6.

Furthermore, an analysis of intron-exon structures was performed. As depicted in Fig. 4, members within the same group exhibited similar intron-exon arrangements. *GhGT64_1* and *GhGT64_5* featured four exons and three introns, *GhGT64_2* and *GhGT64_4* comprised five exons and four introns, and *GhGT64_3* and *GhGT64_6* possessed only one exon.

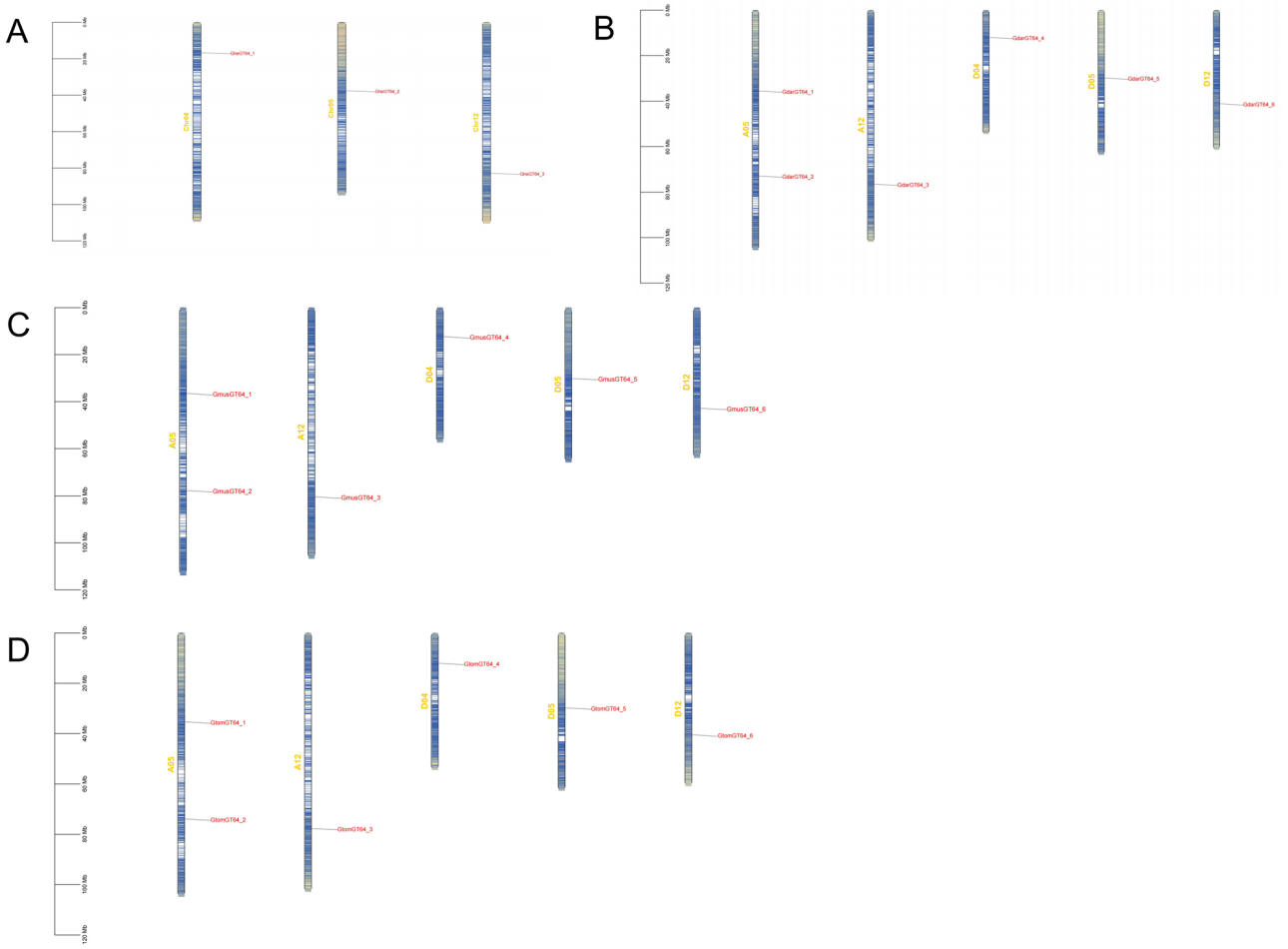


**Fig. 3** The physical locations of the *GT64* genes on the chromosomes, (**A**)-(**D**) represent *G. herbaceum*, *G. darwinii*, *G. mustelinum*, *G. tomentosum*

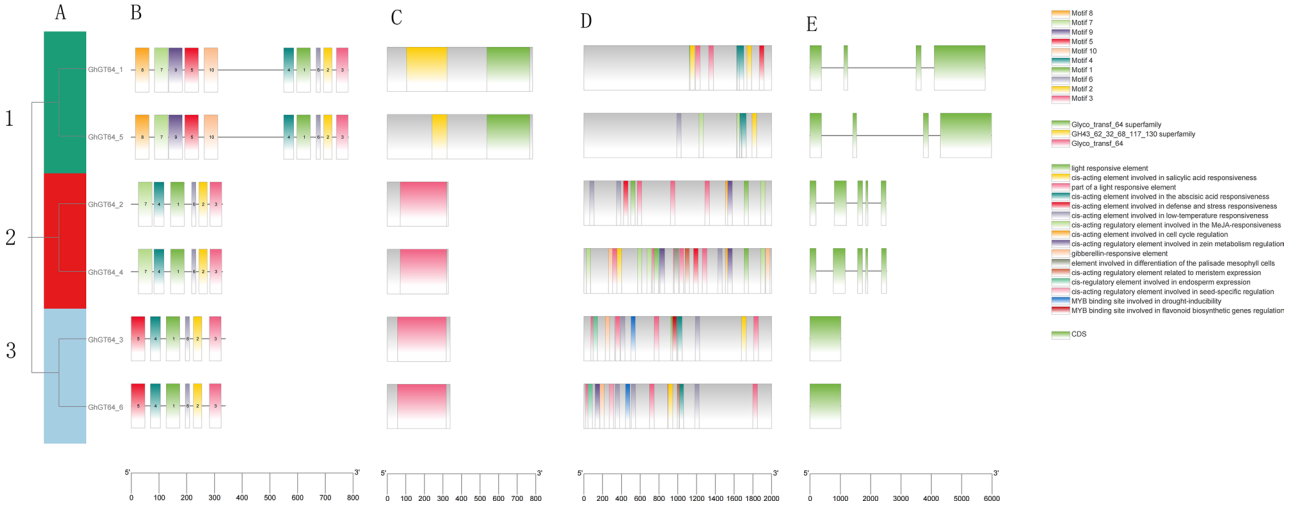


**Fig. 4** The analysis of the *GT64* genes involved a comprehensive investigation of gene structure, motifs, and *cis*-acting elements. The analysis was divided into five main categories: **A**:Phylogenetic tree of upland cotton, **B**:Motif composition and distribution, **C**:Conserved domains of the *GT64* genes, **D**:Visualization of *cis*-acting components, E:Gene structure of *GhGT64s*

We further analyzed the *cis*-acting elements in the promoter regions of the six *GT64* genes in upland cotton. In *G. hirsutum* (Fig. 4), the predicted elements included MYB binding sites related to drought response and flavonoid biosynthesis, light-responsive elements, and various phytohormone-related elements such as those responsive to abscisic acid, salicylic acid, methyl jasmonate, and auxin. Other identified *cis*-regulatory elements were involved in endosperm expression, low-temperature response, zein metabolism regulation, gibberellin response, seed-specific regulation, defense and stress response, palisade mesophyll cell differentiation, meristem expression, and cell cycle regulation. Through promoter analysis, these findings will support the validation of subsequent gene functions.

### Analysis of gene duplication and synteny

In addition, we identified the repeat type of the *GT64* genes in eight cotton genera (Table S2). Among the three diploid cotton species, namely *Gossypium arboreum*, *Gossypium raimondii*, and *Gossypium herbaceum*, all three genes are categorized as Dispersed type. Conversely, in tetraploid cotton species, all genes are classified under the whole-genome duplication or Segmental duplication types.

We conducted multiple collinearity analyses of *GT64* genes in eight cotton genus (Fig. 5). We observed five homologous gene pairs between *G. barbadense* and *G. arboreum*, five homologous gene pairs between *G. hirsutum* and *G. arboreum*, six homologous gene pairs between *G. barbadense* and *G. hirsutum*, six homologous gene pairs between *G. barbadense* and *G. raimondii*, six homologous gene pairs between *G. hirsutum* and *G. raimondii*, 12 homologous gene pairs between *G. darwinii* and *G. hirsutum*, six homologous gene pairs between *G. darwinii* and *G. barbadense*, five homologous gene pairs between *G. darwinii* and *G. arboreum*, six homologous gene pairs between *G. darwinii* and *G. raimondii*, six homologous gene pairs between *G. darwinii* and *G. herbaceum*, 12 homologous gene pairs between *G. darwinii* and *G. mustelinum*, 12 homologous gene pairs between *G. darwinii* and *G. tomentosum*, six homologous gene pairs between *G. mustelinum* and *G. herbaceum*, five homologous gene pairs between *G. mustelinum* and *G. arboreum*, six homologous gene pairs between *G. mustelinum* and *G. raimondii*, 12 homologous gene pairs between *G. mustelinum* and *G. barbadense*, 12 homologous gene pairs between *G. mustelinum* and *G. tomentosum*, 12 homologous gene pairs between *G. mustelinum* and *G. hirsutum*, five homologous gene pairs between *G. tomentosum* and *G. arboreum*, six homologous gene pairs between *G. tomentosum* and *G. raimondii*, 12 homologous gene pairs between *G. tomentosum* and *G. hirsutum*, 12 homologous gene pairs between *G. tomentosum* and *G. barbadense*, six homologous gene pairs between *G. tomentosum* and *G. herbaceum*, three homologous gene pairs between *G. herbaceum* and *G. arboreum*, three homologous gene pairs between *G. herbaceum* and *G. raimondii*, six homologous gene pairs between *G. herbaceum* and *G. hirsutum*, and six homologous gene pairs between *G. herbaceum* and *G. barbadense*, along with three homologous gene pairs between *G. arboreum* and *G. raimondii*. Our hypothesis, based on these observations, is that the GT64 gene family's evolution and gene

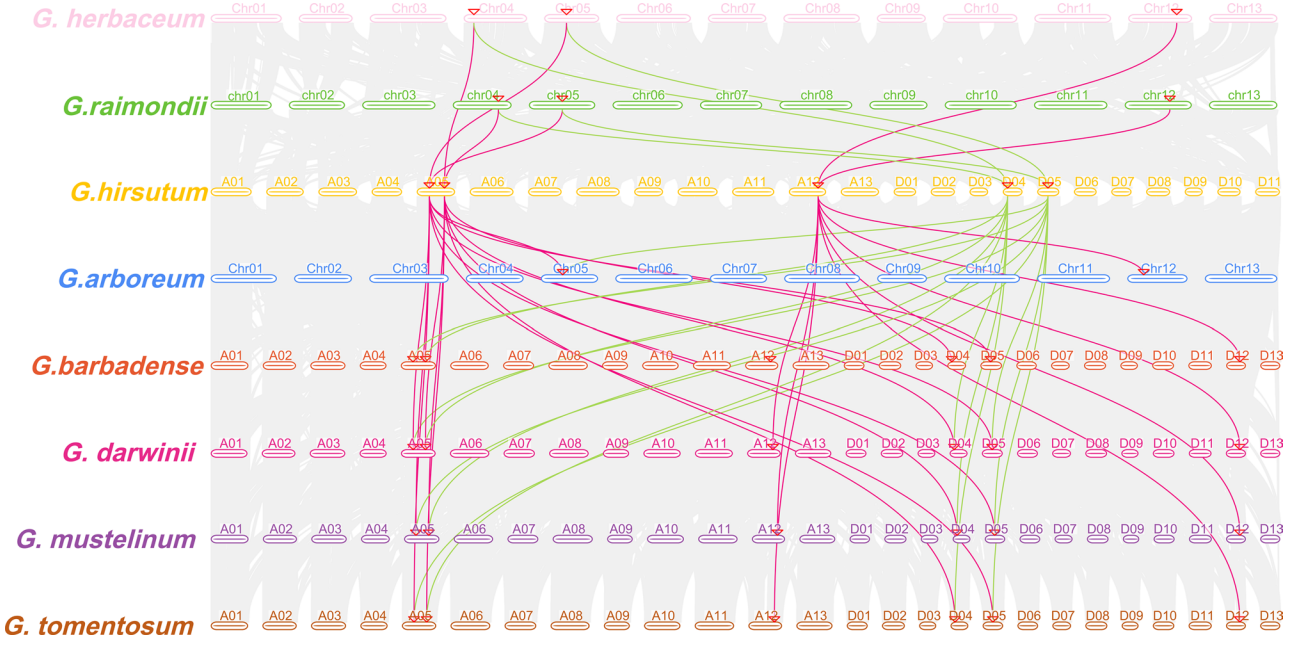


**Fig. 5** Collinearity analysis of eight cotton genera

amplification primarily stem from whole-genome duplication and segmental duplication events.

Subsequently, we conducted collinearity analysis among upland cotton and identified a total of three orthologous/paralogous pairs (Fig. 6B). Within the *G. barbadense* species (Fig. 6A), we identified three orthologous/paralogous pairs. In the other tetraploid cotton species, namely *G. tomentosum* (AD3), *G. mustelinum* (AD4), and *G. darwinii* (AD5), we observed three orthologous/paralogous pairs in each species (Fig. 6C-E). Additionally, no orthologous/paralogous pairs were found in the three diploid cotton species.

### Selection pressure analysis of eight cotton genus

To explore the *GT64* gene differentiation mechanism in cotton polyploid duplication events, we assessed the Ka/Ks ratio to discern selection pressure types on homologous gene pairs (Table S3). The Ka/Ks ratios were computed for 217 homologous gene pairs in eight cotton species individually (Fig. 6F-H). Notably, Ka/Ks ratios below 0.5 were observed between the diploid species *G. raimondii* and *G. arboreum*, as well as between *G. raimondii* and *G. herbaceum*. However, between *G. arboreum* and *G. herbaceum*, one homologous gene pair exhibited a Ka/Ks ratio exceeding 0.5, with another pair exceeding 1, indicating prevalent purifying selection among most homologous gene pairs in diploid cotton species, alongside a few instances of positive selection. Subsequent analyses extended to comparisons between diploid and tetraploid cotton species. Specifically, Ka/Ks ratios remained below 0.5 between *G. herbaceum* and both *G. barbadense* and *G. hirsutum*. Similarly, all homologous gene pairs between *G. herbaceum* and *G. tomentosum* displayed Ka/Ks ratios lower than 0.5. Notably, between *G. herbaceum* and *G. darwinii*, three pairs had ratios below 0.5, while between *G. herbaceum* and *G. mustelinum*, three pairs exhibited ratios below 0.5 and one pair had a ratio exceeding 1. These findings collectively suggest complex evolutionary dynamics involving diverse selection pressures in distinct cotton species and ploidy levels.

In tetraploid species, all homologous gene pairs within *G. barbadense*, *G. hirsutum*, *G. tomentosum*, *G. mustelinum*, and *G. darwinii* exhibited Ka/Ks ratios less than 0.5. Between *G. barbadense* and *G. hirsutum*, *G. barbadense* and *G. mustelinum*, and *G. barbadense* and *G. darwinii*, all homologous gene pairs had Ka/Ks ratios less than 0.5; however, between *G. barbadense* and *G. tomentosum*, two gene pairs had ratios greater than 0.5, while the rest were less than 0.5 between *G. hirsutum* and *G. darwinii*, one gene pair had a ratio greater than 0.5, and one pair had a ratio greater than 1, with the others less than 0.5 between *G. hirsutum* and *G. mustelinum*, one gene pair had a ratio greater than 0.5, while the rest were less than 0.5 between *G. hirsutum* and *G. tomentosum*, two gene pairs had ratios greater than 0.5, with the remaining pairs less than 0.5. Between *G. darwinii* and *G. mustelinum*, one gene pair had a ratio greater than 0.5, and the rest were less than 0.5 between *G. darwinii* and *G. tomentosum*, three gene pairs had ratios greater than 0.5, with

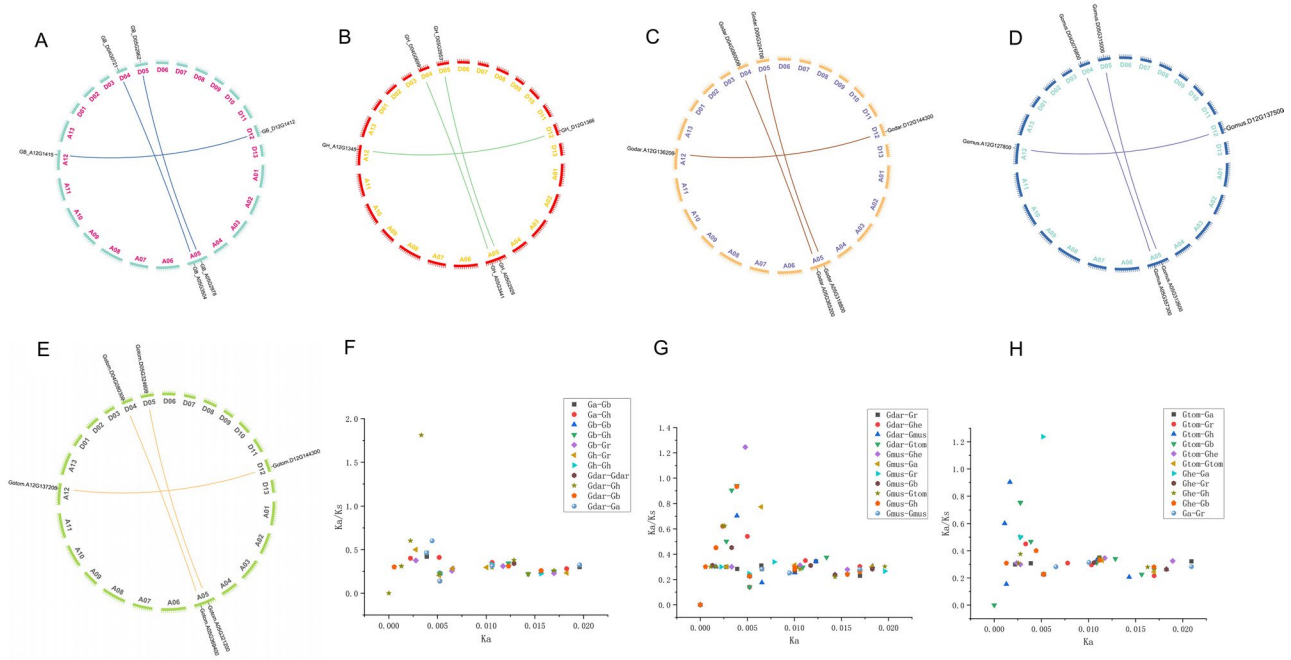


**Fig. 6** Collinearity analysis was conducted for *GT64s* in different cotton specie. (**A**)-(**E**) represent *G. barbadense*, *G. hirsutum*, *G. darwinii*, *G. mustelinum*, and *G. tomentosum*, respectively. (**F**-**H**) Selection pressure analysis was carried out to examine the evolutionary dynamics of the GT64 gene family

the others less than 0.5 between *G. mustelinum* and *G. tomentosum*, two gene pairs had ratios greater than 0.5, while the remaining pairs were less than 0.5. These findings highlight the differential selection pressures and evolutionary dynamics among tetraploid cotton species.

In summary, among the eight cotton species, most *GT64* genes have experienced intense purifying selection throughout evolution, with a few homologous gene pairs showing evidence of positive selection effects.

### Expression profiles of *GT64s* in *G. Hirsutum*

In order to study the expression patterns of GT64 family, we utilized transcriptome data from various tissues of upland cotton [17]. The results indicated (Fig. 7A) that *GhGT64_2* and *GhGT64_4* exhibited higher expression levels compared to other genes in all tissues. Specifically, *GhGT64_2* showed the highest expression in leaf, stem, receptacle, and stamen, followed by *GhGT64_4*, *GhGT64_4* displayed the highest expression in pistil and root, followed by *GhGT64_2* (Fig. 7B). Furthermore, during cotyledon development (Fig. 7C), the expression level of *GhGT64_4* increased gradually with time, reaching its peak at 96 h, followed by a decrease starting from 120 h. In the root development process, both *GhGT64_2* and *GhGT64_4* showed a gradual increase in expression level, reaching their peaks at 120 h. *GhGT64_2* and *GhGT64_4* exhibited a trend of initially increasing and then decreasing expression levels during seed development with time. In the process of fiber development (Fig. 7D), *GhGT64_2* and *GhGT64_4* had higher expression levels in ovules than fibers in the early stages of fiber development, but the opposite was observed in the later stages, where fiber expression levels were higher than ovule expression levels. *GhGT64_2* displayed the highest expression at -1 DPA(days post-anthesis) during the entire fiber development process; *GhGT64_4* exhibited the topmost expression at 20 DPA during development. *GhGT64_5* showed the topmost expression at 35 DPA during ovule development compared to other genes, possibly related to the oil content of cotton seeds. Interestingly, through other studies [25], it was found that *GhGT64_4* showed a gradual increase in expression levels at 2 DPA, 4 DPA, and 6 DPA in fuzz material, but a gradual decrease in fuzzless material, indicating that this gene may be involved in the development of fuzz fiber in upland cotton (Fig. 7E). Subsequently, transcriptome data from high LP material LMY22 and low LP material LY343 [26] revealed that *GhGT64_2* and *GhGT64_4* had higher expression levels in LMY22 compared to LY343 at the same stage of fiber development, suggesting that these two genes may be involved in regulating the changes in lint percentage (LP) during upland cotton fiber development (Fig. 7F).

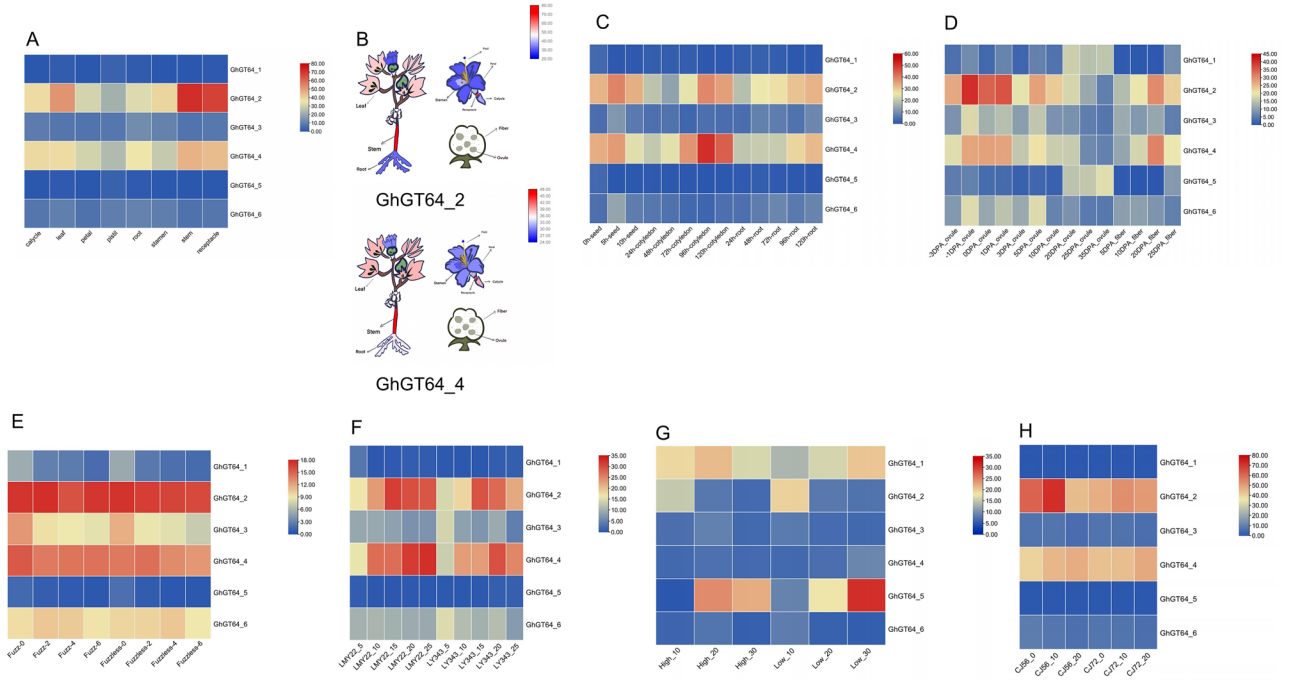


**Fig. 7** Generate a heatmap of the GT64 gene family members based on transcriptome data.(**A**)*GhGT64s* expression profiles in various cotton organs.(**B**) Heatmap showing expression of *GhGT64_2* and *GhGT64_4* in different cotton tissues.(**C**)Expression of *GhGT64s* in different tissues and at different periods of upland cotton.(**D**)*GhGT64s* expression profiles in ovule and fiber at different developmental stages.(**E**) *GhGT64s* expression levels in fuzz and fuzzless materials at various time points. (**F**)Expression levels of *GhGT64s* in ovule and fiber in materials with higher and lower lint percentages at different time points.(**G**)Expression patterns of *GhGT64s* in oil material.(**H**)Expression patterns of *GhGT64s* in CJ56 and CJ72 material

Cottonseed oil has a wide range of applications and at the same time has a certain influence on the quality of cotton [27]. The results showed (Fig. 7G) that *GhGT64_5* exhibited a rapid increase in expression levels at 20 DPA to 30 DPA in the low-oil material, suggesting that this gene may have a negative regulatory effect on the oil content in cotton materials. Cotton is highly susceptible to prolonged waterlogging stress. Then, we are based on transcriptome data published by previous studies [28]. The results indicated that the expression levels of *GhGT64_2* and *GhGT64_4* were higher compared to other genes, suggesting that these two genes play important roles in cotton's tolerance to flood stress and may be key genes for upland cotton's resistance to waterlogging (Fig. 7H).

The development of pigment glands plays a crucial role in cotton, based on previous research [29]. We found that the expression levels of *GhGT64_2* and *GhGT64_4* were higher in the four materials compared to other genes. *GhGT64_2* showed significantly lower expression in Z17YW compared to Z17, while *GhGT64_4* exhibited markedly higher expression in L7XW compared to L7, indicating that these two genes may regulate the development of pigment glands in upland cotton (Fig. 8A). Additionally, based on transcriptome data from defoliant-sensitive materials CIR12 and CCIR50 [30], the results showed that under different temperature treatments, the expression levels of *GhGT64_2* and *GhGT64_4* were higher in both the early and late stages of TDZ (Thidiazuron) treatment compared to the control, indicating that these two genes are involved in cotton's response to TDZ under different temperature conditions (Fig. 8B).

To study the response mechanism of the *GT64* gene to abiotic stress, based on previous studies [17]. The results (Fig. 8C) showed that under salt and polyethylene glycol stress, the expression level of *GhGT64_4* at 12 h was higher compared to other genes, followed by *GhGT64_2*; while under hot and cold stress, the expression level of *GhGT64_2* at 12 h was higher compared to other genes, followed by *GhGT64_4*. *GhGT64_2* and *GhGT64_4* exhibited an initial decrease followed by an increase in expression level under salt stress; under PEG stress, the expression level showed a continuous increase, reaching its peak at 12 h; under hot stress, the expression level displayed an initial increase followed by a decrease. Additionally, based on previous studies [17], it was observed that *GhGT64_2* and *GhGT64_4* exhibited a trend of initial increase, decrease, and subsequent increase in expression level with increasing time after *Verticillium dahliae* infection. Furthermore, compared to other genes, their expression levels increased after inoculation, indicating that these two genes may play a role in cotton's response to *Verticillium dahliae* (Fig. 8D).

### Expression profiles of *GT64s* in *G. barbadense*

Utilizing expression data of the *GT64* genes in different tissues and fiber development stages of *G. barbadense*

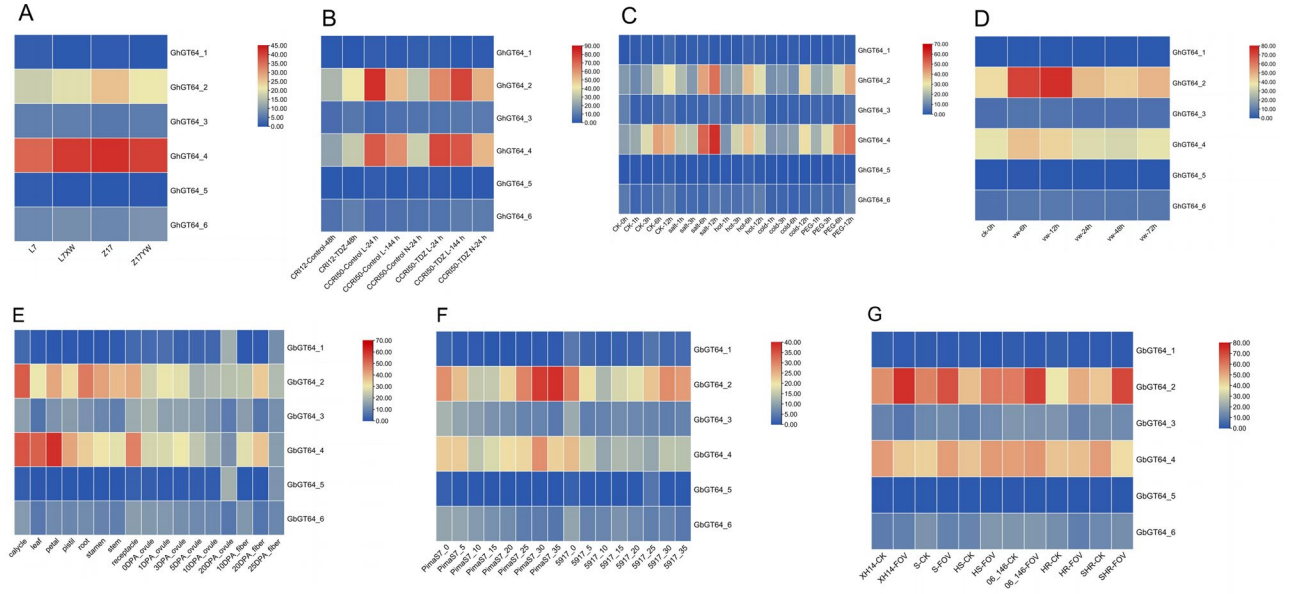


**Fig. 8** Generate a heatmap of the GT64 gene family members based on transcriptome data.(**A**)Demonstration of *GhGT64s* activity in glanded and glandless variants.(**B**)Depiction of *GhGT64s* response in upland cotton to TDZ exposure.(**C**)Display of *GhGT64s* response under extreme temperature, salinity and drought at different time intervals.(**D**)Illustration of *GhGT64s* reaction in *G. hirsutum* when exposed to *Verticillium dahliae* at varying time points.(**E**) Portrayal of *GbGT64s* expression across different tissues and fiber development stages.(**F**)Exposition of *GbGT64s* expressions in *G. barbadense* varieties 5917 and PimaS7 at different fiber development stages.(**G**)Indication of *GbGT64s* reaction in *G. barbadense* under *Fusarium oxysporum f. sp. vasinfectum* stress conditions

[17], we found that *GbGT64_4* exhibited higher expression levels in calycle, pistil, petal, receptacle, and leaf compared to other genes; while *GbGT64_2* demonstrated elevated expression levels in root, stamen, and stem. During ovule development, *GbGT64_2* showed higher expression levels at 1 DPA, 10 DPA, and 20 DPA compared to other genes, while *GbGT64_4* displayed increased expression levels in the beginning of fiber development. In the process of fiber development, *GbGT64_4* showed the greatest expression levels at middle stages of fiber development, followed by *GbGT64_2*; *GbGT64_2* had the topmost expression level at 25 DPA, followed by *GbGT64_4* (Fig. 8E).

The expression levels data of materials with high and low fiber strength of island cotton were also utilized [31]. It was observed that *GbGT64_2* and *GbGT64_4* displayed significant differences in expression levels at 20 DPA, 25 DPA, 30 DPA, and 35 DPA in both materials, indicating the involvement of these two genes in the late-stage fibers development of *G. barbadense*, potentially regulating the quality of *G. barbadense* fiber strength (Fig. 8F). Subsequently, we utilized the expression levels data of disease-resistant and disease-susceptible materials of island cotton [32]. The results depicted (Fig. 8G) that the majority of *GT64* genes showed no significant expression level changes before and after infection. However, The expression levels of *GbGT64_2* and *GbGT64_4* show significant differences in several extreme materials, which may indicate that these two genes play crucial roles in the resistance process of island cotton against FOV.

#### *GhGT64_4* enhances the disease resistance of tobacco

Based on previous work [24], to identify the gene function of *GH_D04G0699* (*GhGT64_4*), We transformed the gene into tobacco or transgenic lines to observe the disease resistance of tobacco.

We selected tobacco lines OE1, OE2, and OE3 with high expression levels for disease resistance assessment. The results showed that plants overexpressing *GhGT64_4* were more resistant to Vd592 than the wild type. After 20 days of inoculation, the disease index of wild type was 76.94, and that of overexpressed plants was 26.02. Compared with wild type, the disease severity of overexpressed plants was significantly reduced (Fig. 9A). By day 20 post-inoculation, the wild-type plants displayed significant leaf necrosis, whereas the overexpressing plants exhibited leaf yellowing without necrosis. Over time, the wild type plants began to exhibit whole-plant necrosis (30 days), whereas the overexpressing plants showed signs of necrosis at 45 days post-inoculation, indicating that *GhGT64_4* enhances tobacco resistance to Vd592 (Fig. 9B-D). qRT-PCR analysis of disease-related genes in tobacco (*NbPR1a*, *NbPR2*, *NbPR9*, *NbPR10a*, *NbLOX*, *NbERF1*) revealed that, except for *NbERF1* and *NbPR9*, in the overexpressing tobacco, the expression levels of the other genes were generally elevated compared to those in the wild type. This indicates that *GhGT64_4* can rapidly respond to *Verticillium dahliae* pathogen stress in the initial stage. Genes associated with the JA pathway, such as *Lox6*, reached their peak expression at 72 h, while genes related to SA synthesis, such as *PR1a*, showed a rapid increase in expression after *V. dahliae* treatment, reaching a level over 10 times higher at 72 h than the control. The PR9 protein, with peroxidase activity, thickens the cell wall by catalyzing lignin synthesis to resist pathogen invasion [33]. This gene showed a rapid increase after *V. dahliae* treatment for 12 h, followed by a decrease. These results demonstrate that *GhGT64_4* can be heterologously expressed in tobacco, by activating disease-related protein genes, the tobacco's tolerance to Verticillium wilt was enhanced (Fig. 9E-J).

#### Validation of *GhGT64_4* in cotton

In addition, VIGS experiments were conducted in cotton, we found that after injection for 15 days, the true leaves and stem veins of cotton exhibited a whitening phenotype, indicating successful gene silencing in cotton (Fig. 10A). qRT-PCR analysis of the target gene silencing efficiency in the experimental plants revealed a significant decrease in the expression levels of the target gene, indicating successful gene silencing in the plants (Fig. 10B-D). After silencing the *GhGT64_4*, the resistance to Verticillium wilt was weakened compared to the control group pTRV2:00. At 15 days post-inoculation with the *V. dahliae* pathogen, plants with silenced *GhGT64_4* showed significantly reduced resistance, with a disease index of 42.92 (Fig. 10E), indicating a marked increase in disease severity compared to pTRV2:00. The results above indicate that *GhGT64_4* is involved in the resistance of upland cotton to Verticillium wilt.

Furthermore, we used qRT-PCR technology to examine the expression levels of resistance-related genes in silenced plants. The results demonstrated (Fig. 10F) that, compared to control plants, pTRV2:*GhGT64_4* plants exhibited significantly reduced expression levels of *Phenylalanine Ammonia Lyase*, *4-Coumarate: CoA Ligase*, *Polyphenol Oxidase*, *Pathogenesis-Related Protein 1*, and *Allene Oxide Cyclase*, while *Chalcone Isomerase*, *Superoxide Dismutase*, *Catalase*, and *Aconitase* showed significantly increased expression levels. These findings suggest that *GhGT64_4* positively regulates the expression of *PAL*, *4CL*, *PPO*, *PR1*, and *AOC* genes, while negatively regulating the expression of *CHI*, *SOD*, *CAT*, and *ACO*, indicating that *GhGT64_4* mainly influences the synthesis of *PAL*, *4CL*, and *AOC*, thereby affecting lignin and JA biosynthesis pathways, ultimately impacting cotton resistance. Following inoculation with Vd592 (Fig. 10G), *4CL*, *POD*, *EDS1*, and *ACO* exhibited significant reductions

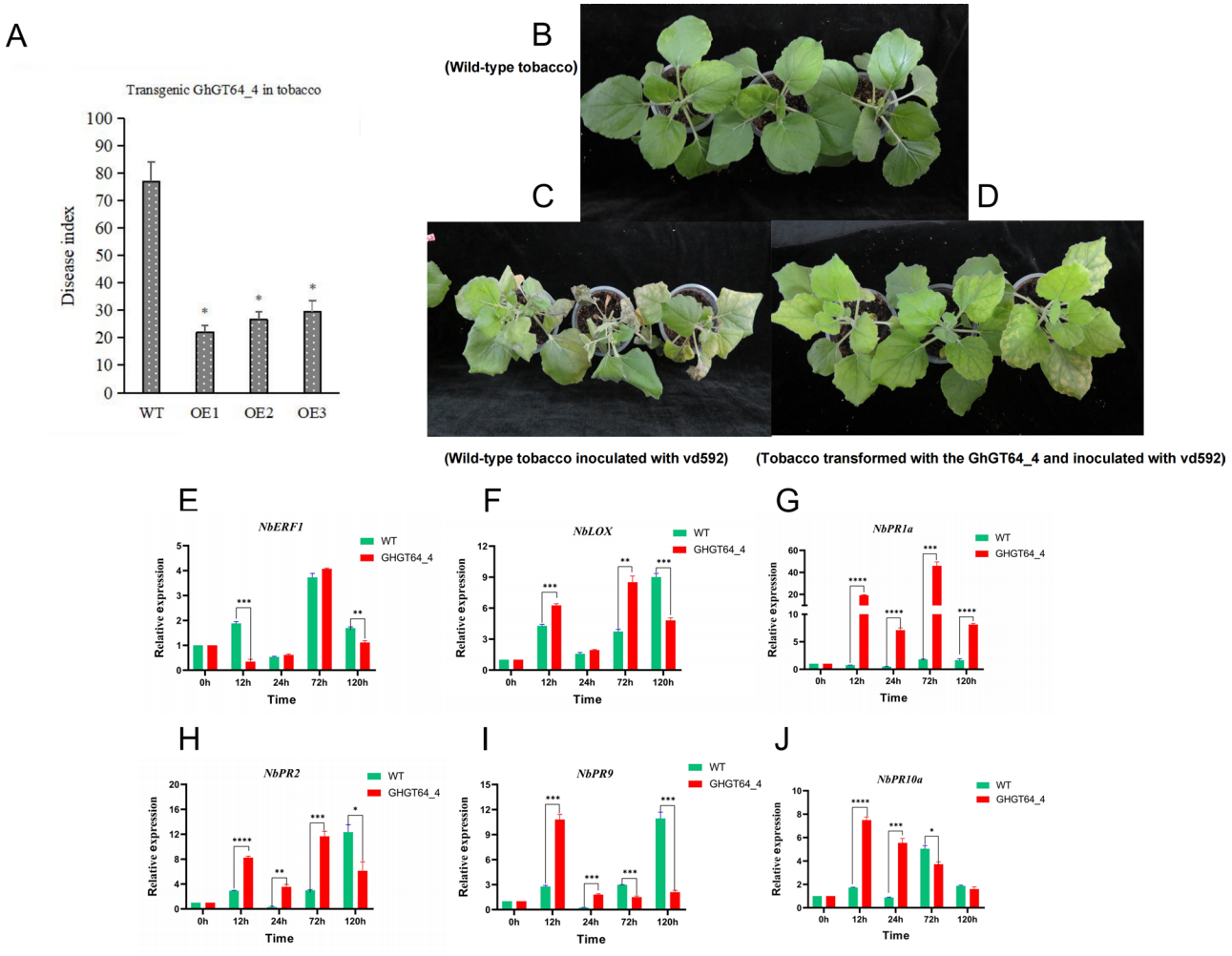


**Fig. 9** Characterization of tobacco resistance in transgenic *GhGT64_4*. (**A**) Assessment of disease resistance index in transgenic and wild type tobacco. (**B**)-(**D**) represent the phenotypes of wild-type tobacco, wild-type tobacco inoculated with vd592, and tobacco transformed with the *GhGT64_4* and inoculated with vd592, respectively. (**E**)-(**J**) Analysis of gene expression levels. Statistical significance was observed in the experimental group compared to the control group at $^{*}P < 0.05$,$^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

compared to pTRV:00, while *SOD* and *AOC* showed significant increases. In addition, the pathogenic bacteria isolated on potato dextrose agar (PDA) showed that a large amount of *V. dahliae* grew in the silenced cotton stems with gene *GhGT64_4*, while no mycelium was observed in the control (Fig. 10H).

Lignin is thought to have a significant impact on shielding cotton plants from *V. dahliae* infection. To validate this finding, we conducted additional measurements of the overall lignin levels. Following inoculation with *V. dahliae*, the stems of TRV:*GhGT64_4* plants exhibited reduced lignin content compared to TRV:00 plants, while exposure to *V. dahliae* resulted in an elevation of lignin levels in the plants (Fig. 10I).

This suggests that the expression levels of certain genes were elevated under the induction of other pathways. Additionally, the interplay or antagonistic effects among JA, SA, and ET pathways could lead to one pathway being enhanced while strongly inhibiting another [34], such as *AOC* and *ACO*. The signaling pathway is a vast and intricate network, where the suppression of one gene may be compensated by others, thus, following induction of Verticillium wilt in cotton, the expression of some disease-resistant genes showed an increase.

### Transcription analysis *GhGT64_4* between *G. Hirsutum* and *G. barbadense*

Although a series of bioinformatics analyses have been conducted on the GT64 gene family and we have gained a basic understanding, their potential role in resistance to Verticillium wilt in upland and island cotton is still unclear. Based on the transcriptome data of 90 published samples [35] (derived from TM-1 and Hai7124 at time points 0 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h pre- and post-inoculation). TM-1 is a variety with susceptibility to the disease, while Hai7124 is disease-resistant. We

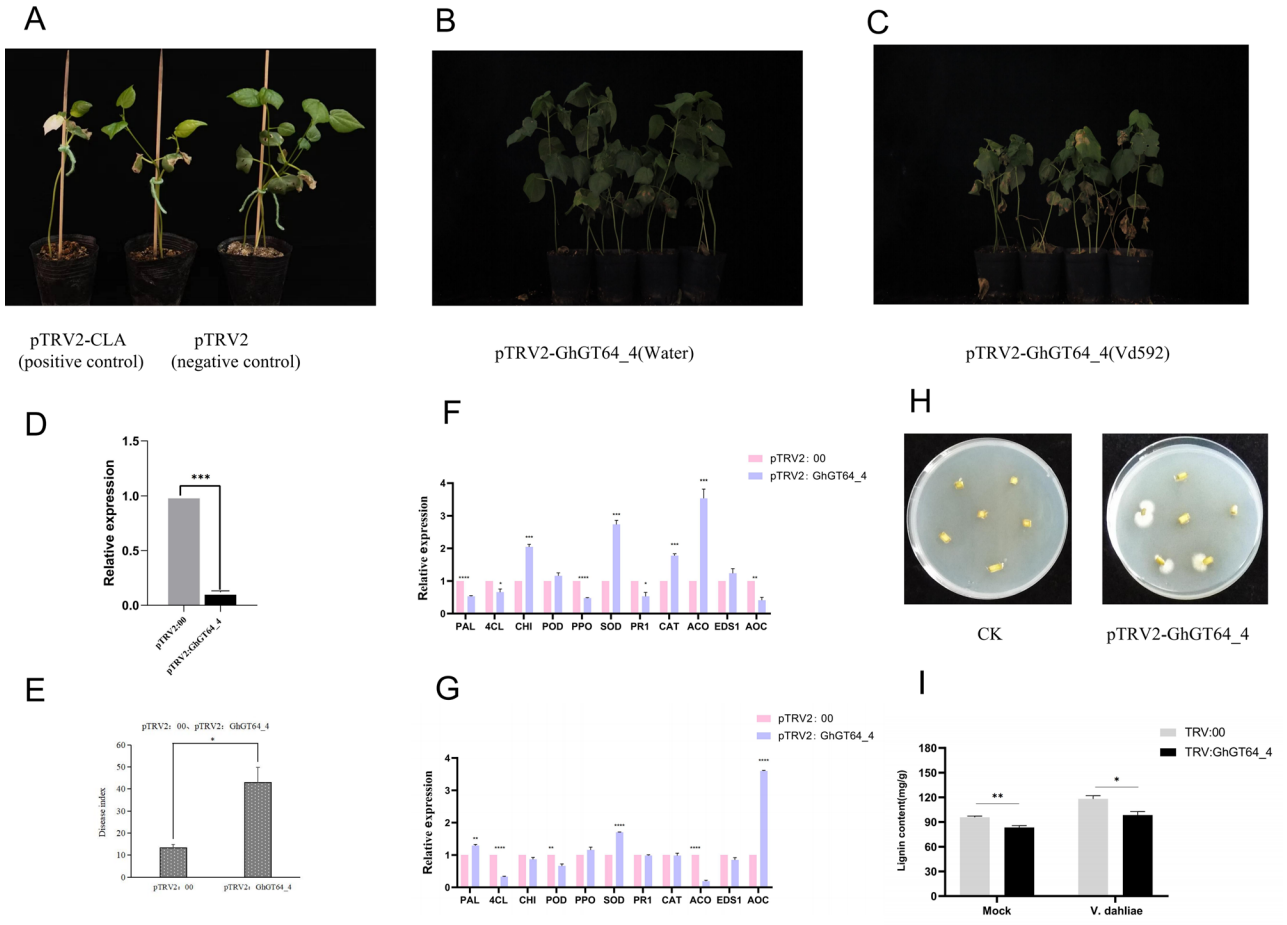


**Fig. 10** Validation of *GhGT64_4* function.(**A**) Plant with albino phenotype (pTRV2-CLA, positive control; pTRV2, negative control). (**B**) The transgenic plants with silenced *GhGT64_4*. (**C**)The transgenic plants with silenced *GhGT64_4* inoculated with vd592. (**D**) VIGS efficiency assessment of *GhGT64_4* in upland cotton. (**E**) Disease resistance index of silenced and normal plants at 15 dpi.(**F**) Expression levels of resistance-related genes in pTRV2:00 and pTRV2: *GhGT64_4* plants. (**G**) Expression levels of resistance-related genes in pTRV2:00 and pTRV2: *GhGT64_4* plants after vd592 inoculation.(**H**) Fungal restoration experiment. (**I**) The lignin content of TRV: 00 and TRV: *GhGT64_4* plants. Statistically significant differences from the control group indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

visualized the expression levels of six *GhGT64* genes in *G. hirsutum* and *G. barbadense* materials (Fig. 11A and B) and found that genes *GhGT64_2* and *GhGT64_4* were highly expressed in both materials. Of note, *GhGT64_4* exhibited consistently higher expression levels in *G. barbadense* compared to *G. hirsutum* from 12 h post-inoculation up to 144 h, indicating its significant role in disease resistance in *G. barbadense* (Fig. 11C).

Subsequently, we conducted Weighted Gene Co-Expression Network Analysis (WGCNA) on 9486 genes with FPKM>10 in *G. hirsutum* and 9357 genes in *G. barbadense*. The result shows that, in the TM-1 material, a total of 19 modules were identified (Fig. 12A), with the MEturquoise module containing the highest number of genes at 3088, and the MElightgreen module having the fewest genes at only 44, averaging 499 genes per module. In the Hai7124 material, 10 modules were identified (Fig. 13A), with the MEturquoise module containing the most genes at 2558, and the MEgrey module containing the fewest genes at 114, averaging 935 genes per module. Core modules were chosen in both materials according to the criteria (|r|>0.50 and $P<0.001$). Notably, the *GhGT64_4* was found in the MEturquoise module in both *G. hirsutum* and *G. barbadense*. Comparison of genes in the MEturquoise module between these two materials revealed 1667 genes that were common to both (Fig. 11D), with 1422 genes and 892 genes unique to *G. hirsutum* and *G. barbadense*, respectively. We performed separate KEGG enrichment analysis on the gene sets of *G. hirsutum* and *G. barbadense*, showing common enrichments in pathways such as Spliceosome, Ribosome, mRNA surveillance pathway, Glutathione metabolism, Valine, leucine, and isoleucine biosynthesis in both materials. In *G. hirsutum*, enrichment was mainly observed in pathways like SNARE interactions in vesicular transport, Oxidative phosphorylation, Sphingolipid metabolism, Ubiquinone, and other terpenoid-quinone biosynthesis (Fig. 11E), whereas in *G. barbadense*,

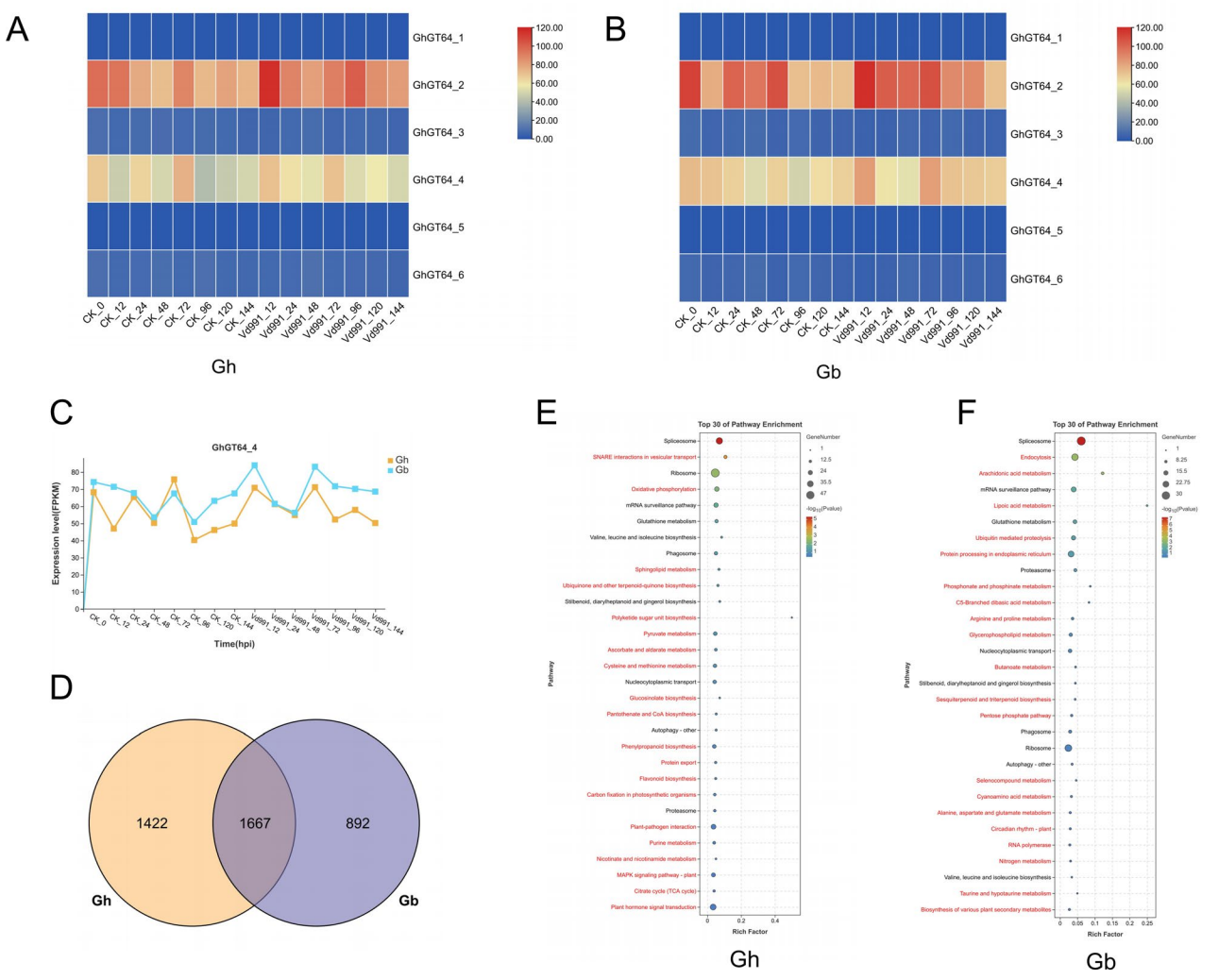


**Fig. 11** Transcriptome data were used to study *GhGT64s* in *G. hirsutum* and *G. barbadense*. (**A**) Expression analysis of *GhGT64s* in *G. hirsutum*. (**B**) Expression analysis of *GhGT64s* in *G. barbadense*. (**C**) Expression analysis of *GhGT64_4* in *G. hirsutum* and *G. barbadense*. (**D**) Number of genes in the MEturquoise module of *G. hirsutum* and *G. barbadense*. (**E**) KEGG pathway enrichment analysis of the MEturquoise module in *G. hirsutum*. (**F**) KEGG pathway enrichment analysis of the MEturquoise module in *G. barbadense*

enrichment was primarily seen in pathways such as Endocytosis, Arachidonic acid metabolism, Lipoic acid metabolism, Ubiquitin-mediated proteolysis, and Sesquiterpenoid and triterpenoid biosynthesis (Fig. 11F).

Furthermore, we observed that in *G. hirsutum*, the MEturquoise module was significantly positively correlated with 144 h post-inoculation (Fig. 12B), while in *G. barbadense*, the MEturquoise module was significantly positively correlated with 72 h post-inoculation (Fig. 13B), indicating that *GhGT64_4* may have initiated its immune response against Verticillium wilt in *G. barbadense* earlier than in *G. hirsutum*. We selected 150 genes with weight values greater than 0.02 from the MEturquoise module in each material as potential interacting partners with *GH_D04G0699* (Table S4). To investigate the potential role of the *GH_D04G0699* interaction network (Figs. 12C and 13C), we conducted KEGG pathway analysis on the set of 150 genes in each sample material (Fig. 12D). In both materials, the enriched pathways for these genes were primarily related to Plant-pathogen interaction, Endocytosis, alpha-Linolenic acid metabolism, and MAPK signaling pathway. We hypothesize that during infection with *Verticillium dahliae*, the two cotton species may employ metabolic pathways and signaling pathways to resist the disease. Interestingly, in *G. barbadense* (Table S5), processes such as RNA degradation and Ubiquitin-mediated proteolysis played significant roles in combating Verticillium wilt, unlike in *G. hirsutum* (Fig. 13D).

### Expression analysis of *GT64s* in different cotton varieties

To examine the level of *GT64s* expression under different stress conditions in various cotton varieties. Through the previous studies of expression and *cis*-acting elements,

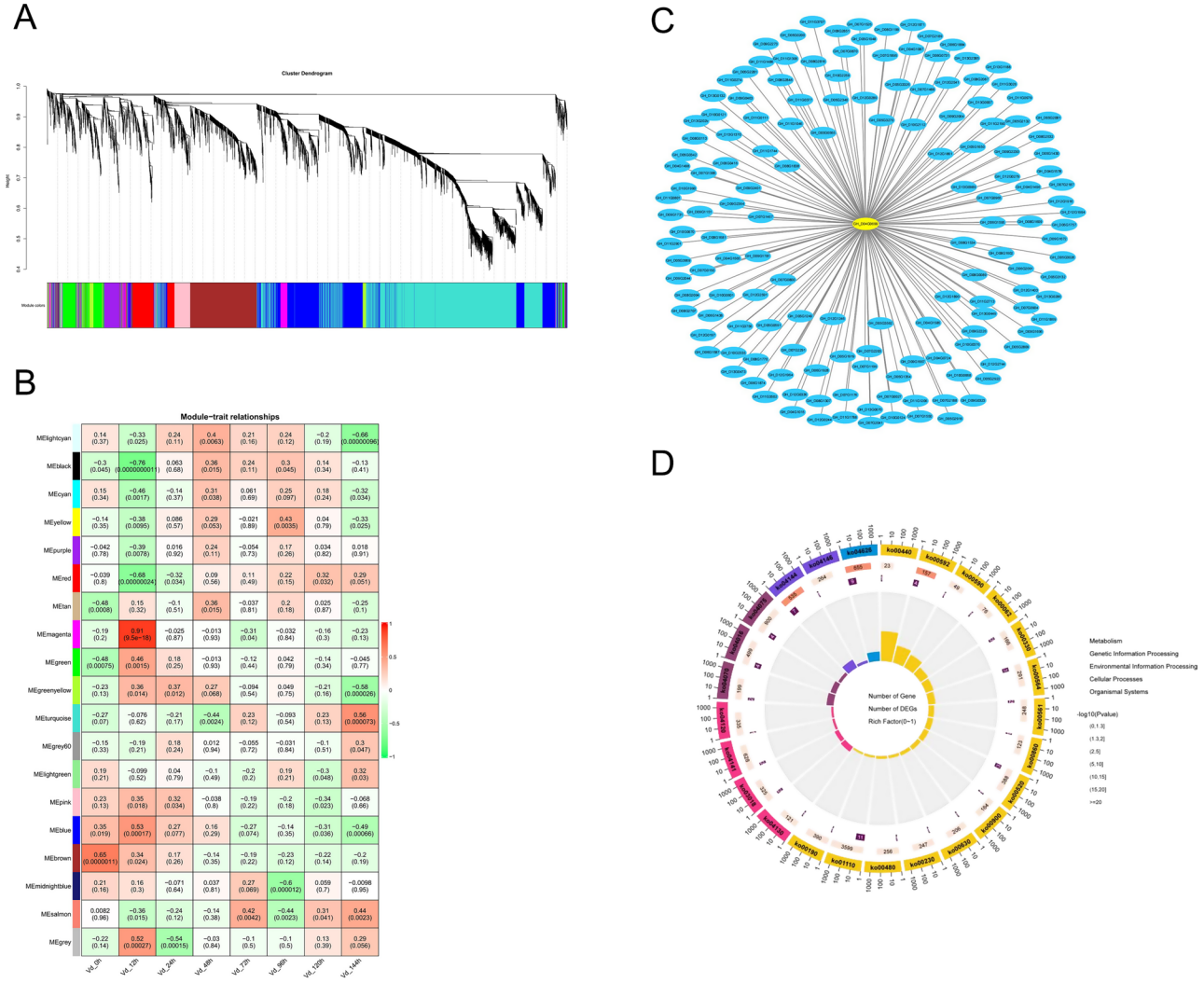


**Fig. 12** WGCNA in *G. hirsutum*. (**A**) Gene clustering analysis outcomes from WGCNA on transcriptome data in *G. hirsutum*. (**B**) Heatmap illustrating correlations between modules and traits. (**C**) Development of the comprehensive network for *GhGT64_4*. (**D**) Perform KEGG pathway enrichment analysis on 150 genes that interact with *GhGT64_4*

we propose that *GhGT64_1*, *GhGT64_2*, *GhGT64_4*, *GhGT64_5* may play roles in responding to various biotic and abiotic stress factors, fuzz fiber development, and modulation of oil content in cottonseeds.To further elucidate these roles, we conducted expression pattern analyses on selected *G. hirsutum* and *G. barbadense* cultivars to monitor the expression dynamics of these genes across different cotton species and temporal stages.

Initially, *GhGT64_1* and *GhGT64_5* were selected for fluorescence quantitative PCR analysis in cottonseeds with varying oil content, specifically low oil content from Emian22 and high oil content from 3 to 79, as depicted in Fig. 14A. Over the course of cottonseed maturation from 10 DPA to 30 DPA, a time-dependent increase in the expression levels of both genes was observed. Particularly noteworthy was the significant divergence in expression levels at 20 DPA and 30 DPA between the two varieties, suggesting a potential regulatory role of these genes in cottonseed oil production.

Subsequently, *GhGT64_2* and *GhGT64_4* were investigated via fluorescence quantitative PCR in fuzzless series material, as illustrated in Fig. 14B. While *GhGT64_2* exhibited consistent expression levels, *GhGT64_4* displayed significant differences in expression at 1 DPA and 3 DPA between the two variants. Notably, at 3 DPA, the expression of *GhGT64_4* was higher in the fuzzless mutant, indicating a potential positive regulatory role in fuzz fiber development in *G. hirsutum*.

Following inoculation with *V. dahliae*, the transcription levels of these genes were markedly induced at specific time points in both resistant and susceptible variants, as shown in Fig. 14C. *GhGT64_2* and *GhGT64_4* displayed distinct expression patterns, with peak levels at different time points post-inoculation, suggesting their

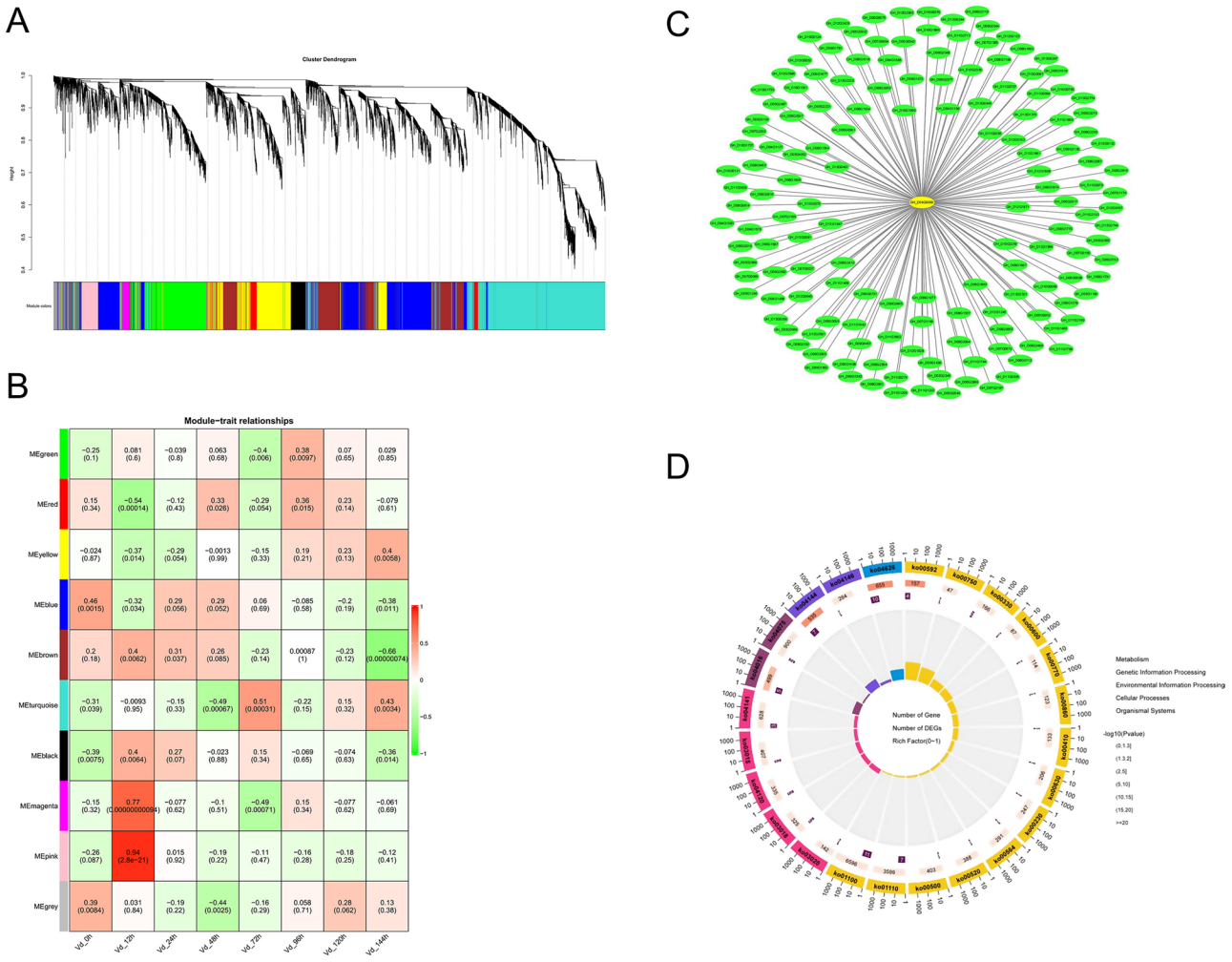


**Fig. 13** WGCNA in *G. barbadense*. (**A**) Gene clustering analysis outcomes from WGCNA on transcriptome data in *G. barbadense*. (**B**) Heatmap illustrating correlations between modules and traits. (**C**) Development of the comprehensive network for *GhGT64_4*. (**D**) Perform KEGG pathway enrichment analysis on 150 genes that interact with *GhGT64_4*

involvement in the response to *V. dahliae* invasion in cotton plants.

Upon exposure to PEG-induced drought stress, the transcription levels of *GhGT64_2* and *GhGT64_4* indicated a potential contribution to *G. hirsutum's* response to drought conditions, as depicted in Fig. 14D. The genes exhibited a dynamic expression pattern of initial downregulation, subsequent upregulation, and final downregulation, highlighting their potential role in drought stress response.

Subsequent examination of salt stress response in *G. hirsutum* variants Xinluzao26 (resistant to salt stress) and Xinluzhong30 (susceptible to salt stress) revealed that *GhGT64_2* and *GhGT64_4* may participate in the response to salt stress conditions, as shown in Fig. 14E. Both genes exhibited similar expression dynamics under salt stress, there are significant differences between the two extreme materials at specific points in time, suggesting their involvement in salt stress response.

The expression profiles of *GbGT64_2* and *GbGT64_4* were examined in *G. barbadense*, specifically in the FOV-resistant cultivar 06-146 and the FOV-susceptible cultivar Xinhai14, under FOV stress conditions, as presented in Fig. 14F. The results indicated contrasting roles of these genes in response to FOV stress, with significant differences in expression levels at various time points, underscoring their potential involvement in different stress conditions.

## Discussion

Verticillium wilt has emerged as a significant challenge facing high and stable cotton production, often described as the "cancer" of cotton. In recent years, consecutive plantings have led to a severe invasion of Verticillium wilt in the cotton industry, resulting in a substantial impact

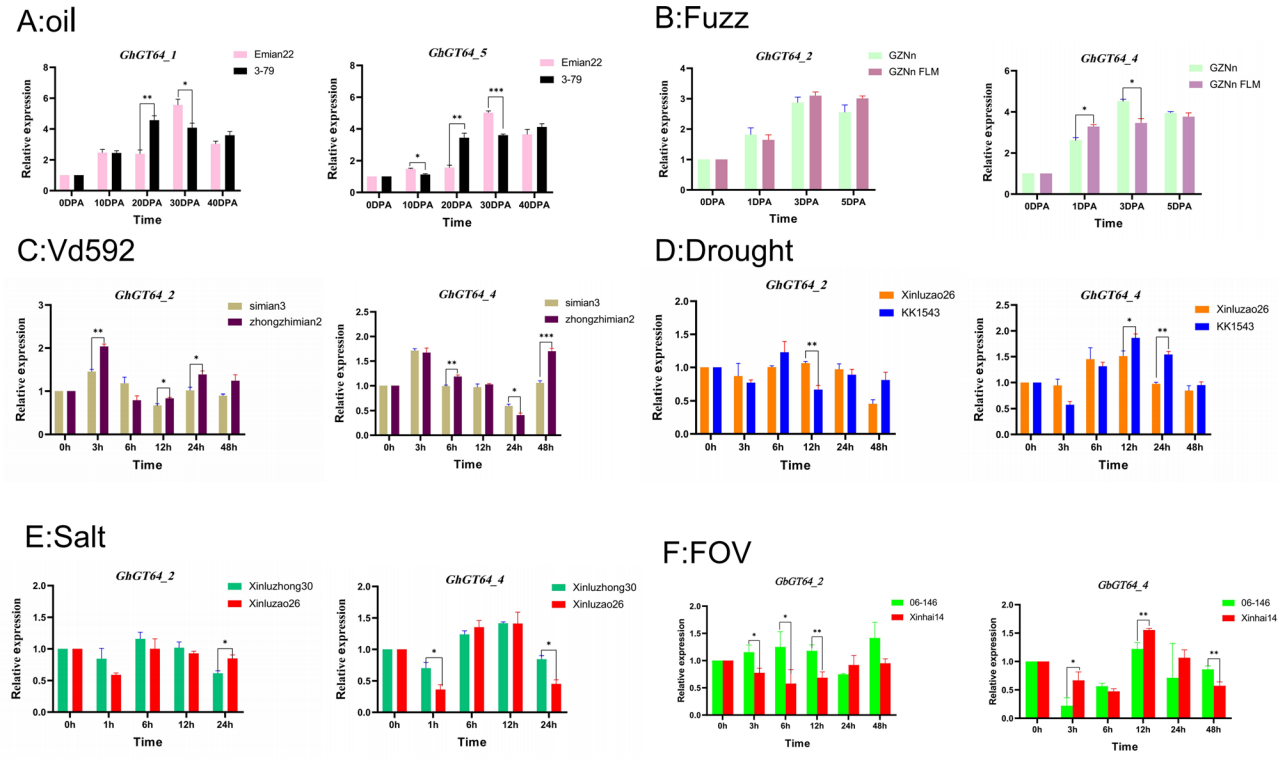


**Fig. 14** qRT-PCR analysis of the *GT64s* in upland cotton and island cotton. (**A**) Expression analysis of *GhGT64s* in different oil materials. (**B**) Expression patterns of *GhGT64s* during fiber development. (**C**) Expression of *GhGT64s* after inoculation with vd592. (**D**) Expression patterns of *GhGT64s* under PEG stress. (**E**) Expression patterns of *GhGT64s* under salt stress. (**F**) Expression patterns of *GbGT64s* under FOV stress. The error bars indicate the means of three technical replicates ± standard errors. Statistically significant differences from the control group are denoted as $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

on yield. However, with the continuous advancement of genetic engineering, enhancing cotton's disease resistance has become an extremely daunting and complex task. Research indicates that a single reference genome is insufficient to capture the diversity of species [36–38]. By analyzing the genomes of eight cotton species, a pan-genome has been constructed [39]. Hence, we performed identification and characterization of the GT64 gene family in eight distinct cotton species to explore potential strategies for improving disease resistance in cotton. In recent years, various families in cotton have been studied, such as *GBSOT* [40], *GhGABA* [41], *GhIFR* [42], *GhGGPS* [43], *GhTBL* [44], *GhDREB* [45], *GhSBT* [46], *GhLOG* [47], *GhGATL* [48], *GhMDVL* [49], and *GhANN* [50].

Based on previous studies, we found a candidate gene *GH_D04G0699* (*GhGT64_4*) for resistance to verticillium wilt in cotton [24]. Subsequently, we performed bioinformatics analysis of this gene in eight cotton species, including a series of systematic analysis of gene families and analysis of expression patterns under various conditions. Furthermore, we studied the function of the *GhGT64_4* gene under *Verticillium dahliae* stress. Subsequently, we preliminarily explored the response mechanisms of the *GhGT64_4* to pathogen invasion in *G. hirsutum* and tobacco using VIGS and heterologous overexpression techniques, and gained initial insights into the pathways and interaction networks of the *GhGT64_4* through the WGCNA method.

### Basic analysis of GT64 family members

We performed phylogenetic and physicochemical analysis using *GT64* gene sequences from eight *gossypium* (Fig. 1), and then visualized the physical locations of the family members on the chromosomes. Interestingly, in five tetraploid cotton species, the number of *GT64* genes was double that of diploid cotton species. This further confirms the two diploid cotton ancestors of tetraploid cotton [20].

In addition, we conducted a multiple collinearity analysis of the GT64 gene family in eight cotton species, and the results indicated that gene expansion in the GT64 gene family evolution was mainly attributed to whole-genome duplication events and segmental duplication events.

Furthermore, we conducted Ka/Ks analysis on diploid and tetraploid cotton species, and the results showed that most *GT64* genes underwent significant purifying selection during their evolutionary history. However, a few homologous genes exhibited signs of positive selection,

indicating their fast evolutionary rate and potential significance in recent species evolution (Fig. 6).

### Investigation into expression profiles of GT64 gene family members

The expression patterns of genes are closely associated with their functions. Through transcriptome data analysis (Fig. 7), we found that in *G. hirsutum* tissues, *GhGT64_2* and *GhGT64_4* exhibited higher expression levels across all tissues compared to other genes. During fiber development, *GhGT64_2* and *GhGT64_4* showed expression levels were higher in ovules compared to fibers during the early stages of fiber development, but this pattern reversed in the later stages, with higher expression levels in fibers than in ovules. Interestingly, we discovered that *GhGT64_4* may be involved in the development of fuzz fibers in upland cotton, while *GhGT64_2* and *GhGT64_4* may regulate changes in lint percentage (LP) during fiber development. Additionally, we found that *GhGT64_5* may negatively control the oil content in cotton materials. Furthermore, *GhGT64_2* and *GhGT64_4* play crucial roles in upland cotton's tolerance to flooding abiotic stress and may be key genes involved in flood resistance, promoting the formation of pigment glands in *G. hirsutum*. Moreover, we observed that *GhGT64_2* and *GhGT64_4* are involved in cotton's response to TDZ treatment under both normal and low temperatures. Additionally, we found that *GhGT64_2* and *GhGT64_4* participate in responses to salt, PEG, heat, and cold stress, as well as in early and late responses of cotton to Verticillium wilt.

We also utilized the transcriptome data of sea island cotton to discover that *GbGT64_2* and *GbGT64_4* not only participate in the later stage fiber development of sea island cotton (Fig. 8), but may also control the fiber strength characteristics of sea island cotton. Additionally, *GbGT64_2* and *GbGT64_4* may play a crucial role in the resistance process of sea island cotton against FOV.

Besides the previously mentioned use of transcriptome data, we also conducted predictions on the *cis*-acting elements of six *GT64* genes in *G. hirsutum*. These elements encompass the MYB binding site that plays a role in drought-inducibility and *cis*-acting elements related to plant hormones such as elements responsive to abscisic acid, salicylic acid, MeJA, and auxin (Fig. 4D). Our findings show that *GhGT64_4* has the most *cis*-acting elements, whereas *GhGT64_1* has the least. By integrating the analysis of transcriptome data and promoter *cis*-acting element assessment, we managed to gain further insights into the matter, we selected *GhGT64_1*, *GhGT64_2*, *GhGT64_4*, *GhGT64_5*, as well as *GbGT64_2*, *GbGT64_4* for qRT-PCR testing under various biological and abiotic stress conditions, and extreme materials with specific traits (Fig. 14). The results indicate that *GhGT64_2* and *GhGT64_4* may influence the formation of fuzz fibers in *G. hirsutum* and possibly respond to salt stress, drought stress, and treatment with Verticillium wilt. *GhGT64_1* and *GhGT64_5* may be involved in the accumulation of cottonseed oil content. In *G. barbadense*, *GbGT64_2* and *GbGT64_4* may participate in the response to FOV treatment.

We know that the defense system established by Hai7124 is rapid, relatively continuous, extensive, and high-intensity, while the disease defense model adopted by TM-1, its core resistance-related process is delayed [35]. Furthermore, utilizing transcriptome data [34], we observed that in *G. hirsutum*, the MEturquoise module was significantly positively correlated with post-inoculation at 144 h, while in *G. barbadense*, the MEturquoise module was significantly positively correlated with post-inoculation at 72 h. This may suggest that *GhGT64_4* initiated immune response to Verticillium wilt earlier in *G. barbadense* compared to *G. hirsutum* (Figs. 12 and 13).

### Functional verification of *GhGT64_4* in *G. Hirsutum*

*Glycosyltransferases* (GTs) are enzymes that facilitate the movement of sugar elements from activated donor molecules to specified acceptor molecules, and they are vital in the formation of the plant cell wall [51]. Wu et al. [52] found that the *glycosyltransferase UGT76B1* is involved in plant defense responses by glycosylating 2-hydroxy-3-methylpentanoic acid, leading to upregulation of SA-related gene expression in mutants, inducing expression of related defense genes, and subsequently triggering plant defense responses. Qin et al. [53] discovered that the *glycosyltransferase UGT76D1* can glycosylate 2,3-DHBA and 2,5-DHBA in the SA metabolic pathway, resulting in SA accumulation, formation of hypersensitive reactions, and involvement in plant immunity, distinct from the regulation of SA synthesis in the above-mentioned studies. In this study, inhibiting the expression of *GhGT64_4* led to a significant decrease in the expression of genes *PAL* and *4CL* involved in the phenylpropanoid pathway, as well as a significant decrease in the expression of *POD* and *AOC* related to JA synthesis. Some phenylpropanoids can polymerize to form defense structures such as lignin, while phenolic compounds (e.g., ferulic acid or coumaric acid) are associated with esterification reactions in cell walls, suggesting that *GhGT64_4* is involved in regulating the synthesis pathways of lignin and JA-related genes. Differential regulation of hormone pathways may be related to the parasitic modes of crops and pathogens. Following induction by *Verticillium dahliae*, the expression of genes associated with ET synthesis, such as *ACO*, significantly increased; some studies suggest an antagonistic relationship between ET and JA, as ET can inhibit the expression of key genes in the JA pathway, such as *THI2.1* and *VSP* [54]. After inhibiting the

expression of the *GhGT64_4*, the expression of *AOC* significantly decreased, while the expression levels of genes associated with ET synthesis increased significantly. Following treatment with *V. dahliae*, the expression of *AOC* may be induced by other genes, leading to a significant increase in expression, while the expression of *ACO* is partially inhibited, resulting in decreased expression. Chitinase (CHI) gene expression decreased after silencing the *GhGT64_4* and significantly decreased after *V. dahliae* induction, suggesting that inhibiting *GhGT64_4* expression weakens *CHI* synthesis; after induction by the pathogen, *CHI* is gradually consumed, and synthesis related to *CHI* is hindered after gene silencing, leading to a significant decrease in expression. *PR1* and *CHI* exhibit similar mechanisms. In addition, compared with control plants, lignin content of silenced plants increased significantly, indicating that this gene may resist the invasion of pathogens through lignin. In conclusion, the regulation of disease resistance signals is a complex and expansive network system, and further research and exploration are required to elucidate how *GhGT64_4* directly or indirectly affects the expression of *PAL*, *4CL*, *PR1*, *CHI*, *AOC*, and *ACO*.

Although the tobacco plant overexpressing the *GhGT64_4* does not exhibit immunity or resistance to *V. dahliae*, it can enhance resistance to Verticillium wilt and delay wilting, thus still holding positive implications for production. By analyzing the expression patterns of six genes (*NbPR1a*, *NbPR2*, *NbPR9*, *NbPR10*, *NbLOX*, *NbERF1*) in tobacco using qRT-PCR technology, it was found that, except for *NbERF1* and *NbPR9*, the expression levels of the other genes were higher in the transgenic tobacco than in the wild type, suggesting their responsiveness to *V. dahliae* stress. The PR1a protein decomposes fungal cell walls or induces separation of pathogenic cells, inhibiting their growth and development, while the PR2 protein exhibits β-1,3-glucanase activity; PR9 rapidly increases 12 h after *V. dahliae* infection, possibly due to the induction of lignin-related genes in response to Verticillium wilt, thickening the cell wall. The wild-type tobacco showed a slight increase in PR9 at a later stage, reaching its peak at 120 h. In the validation results of *GhGT64_4* silencing, genes involved in the phenylpropanoid pathway, *PAL* and *4CL*, significantly decreased, indicating their involvement in the formation of defense structures such as lignin, further suggesting the potential role of *GhGT64_4* in regulating lignin synthesis; the *PR1a* gene related to SA synthesis exhibited a rapid increase after *V. dahliae* treatment, reaching its peak at 72 h, more than 10 times higher than the control, suggesting its potential role in regulating SA synthesis, consistent with previous studies [55, 56]. In contrast to the VIGS validation results, the expression of *AOC* related to JA synthesis significantly decreased, while *EDS1* related to SA synthesis showed no significant change. Disease resistance pathways mediated by genes vary among different crops. The PR10 protein possesses ribonuclease activity and defends against pathogen invasion through phosphorylation, playing a crucial role in plant disease resistance [57]. The indication that disease-resistant-related genes are expressed implies that these genes have the ability to stimulate the production of proteins related to disease resistance, thereby increasing tobacco's tolerance to *V. dahliae*.

## Conclusion

The study provided a detailed analysis of the GT64 gene family in eight species of the cotton genus, including bioinformatics analysis and expression pattern elucidation using RNA-seq data for the first time. Through heterologous overexpression in tobacco and virus-induced gene silencing (VIGS) experiments, it confirmed the involvement of *GhGT64_4* in the process of resistance to Verticillium wilt in cotton. Then, it was discovered that *GhGT64_4* initiated an immune response against *Verticillium dahliae* earlier in *G. barbadense* compared to *G. hirsutum*. Furthermore, qRT-PCR analysis indicated that some *GT64* genes may play roles under various biological and abiotic stress conditions. This study lays a foundation for further study of the role of *GT64* gene in cotton.

## Materials and methods

### Identification GT64 gene family members in eight cotton species

Download the reference genomes of eight cotton species from the CottonFGD database (https://cottonfgd.org/) [58]. Download the seed file PF09258 of the GT64 protein from the Pfam database (http://pfam.xfam.org/) [59], and use HMMER 3.0 software to identify the amino acid sequences of the eight cotton species containing the conserved GT64 domain (*E* value < 0.0001), with further validation by NCBI-CDD.

Analyze the relative molecular weight, theoretical isoelectric point of GT64 family members using online tool (https://web.expasy.org/compute_pi) [60].

### Phylogenetic analysis of GT64 family protein sequences

To investigate the evolutionary relationship of *GT64* genes in eight cotton species, the obtained genes were subjected to multiple sequence alignment using MEGA (MEGA7) and ClustalW [61]. After aligning the sequences, an evolutionary tree was built using the Maximum Likelihood (ML) method with a Bootstrap value of 1000, utilizing the comparative alignment results.

### Gene structure and conserved motif identification in upland cotton

The gene structure information of the GT64 gene family, including open reading frame (ORF) length, protein length, and number of exons, were obtained from the Cotton Functional Genes Database (https://cottonfgd.org/). Analyze the conserved motifs of family members using TBtools-II software (v2.0125), then visualize the results obtained using TBtools-II software (v2.0125) [62].

### Chromosomal mapping of GT64 gene family

Plot the chromosomal distribution of cotton *GT64* based on its gene position information and the length of cotton chromosomes using TBtools-II software (v2.0125) [62].

### Analysis of the expression profiles and *cis*-regulatory elements of GT64 gene family

Transcriptome data were downloaded from the NCBI database (PRJNA248163) and the TBtools-II software (v2.0125) was used to visualize the expression levels of target genes in different cotton species [62].

Use TBtools-II software (v2.0125) to obtain the 2000 bp sequence upstream of the start codon of the *GT64* gene, then submit the obtained sequence to the PlantCARE database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) to predict the *cis*-acting elements present [62].

### Synteny analysis of GT64 gene family

Blast analysis was performed on protein sequences from eight cotton species, and the obtained data was compared using the MCScanX software (V1.1). Further visualization and analysis were carried out using the TBtools-II software (v2.0125) [62, 63].

### Calculation of selective pressure

To assess the selection pressure experienced by the GT64 gene throughout its evolutionary history, the TBtools-II software (v2.0125) was employed to determine the nonsynonymous substitution (Ka) and synonymous substitution (Ks) rates of the repetitive genes [62].

### WGCNA of *GhGT64s* in *G. Hirsutum* and *G. barbadense*

By utilizing transcriptome data from two cotton varieties [35], WGCNA analysis was performed with the WGCNA package (version 4.1.1). Upon threshold selection, a power function was applied to the scale-free relationship matrix with $\beta=5$ for *G. hirsutum* and $\beta=7$ for *G. barbadense*, resulting in an unscaled adjacency matrix. A minimum module gene count of 30 was specified [64].

### Functional Enrichment Analysis in KEGG and construction of interaction networks

The TBtools-II software (v2.0125) was used for KEGG enrichment analysis of the target gene with statistical thresholds set at $P<0.01$ and $Q<0.05$. Pearson correlation coefficient was calculated to construct a gene interaction network, which was visualized using TBtools-II software (v2.0125) [62].

### Silencing and heterogenic overexpression of *GhGT64_4*

The function of *GhGT64_4* was validated using virus-induced gene silencing (VIGS) technology [65]. The silencing fragment was designed using the SGN VIGS Tool (https://vigs.solgenomics.net/) and constructed into the silencing vector pTRV2, targeting the *GhGT64_4* gene (Table S6). After inoculation, the plants were grown under controlled conditions and then inoculated with Vd592 15 days later, following standard procedures. Each treatment group included at least 30 seedlings and was repeated three times. The disease index of each seedling was recorded [66].

*GhGT64_4* was amplified using seamless cloning primers and high-fidelity enzymes, and subsequently ligated into the plant expression vector PHB following digestion with *Hind III* and *XbaI* enzymes. The resulting construct was then transformed into *Escherichia coli* for plasmid extraction, and subsequently transferred into *Agrobacterium* for delivery into tobacco plants [67].

### Plant material and qRT-PCR analysis

The seeds of *Nicotiana benthamiana* and upland cotton varieties Zhongzhimian 2, Simian 3, Emian22, GZNn, GZNnFLM, KK1543, Xinluzao 26, Xinluzhong 30, as well as island cotton varieties 3–79, 06-146, Xinhai14, are preserved at the Key Laboratory of Agricultural Biotechnology of Xinjiang Agricultural University.

Following knockdown of *GhGT64_4*, RNA was isolated from the roots of Zhongzhimian 2 to assess the expression of relevant resistance genes. Similarly, RNA extraction was carried out from the leaves of tobacco plants overexpressing certain genes to analyze the levels of associated disease resistance genes.

Ovule samples were collected at five developmental stages (0, 10, 20, 30, 40 DPA) from 3 to 79 and Emian22. Ovule and fiber samples were also gathered at four stages (0, 1, 3, 5 DPA) from fuzz and its fuzzless mutant materials for expression profiling.Furthermore, gene expression analysis was conducted under drought and salt stress conditions using materials KK1543, Xinluzao 26, Xinluzhong 30, and Xinluzao 26. Seeds of KK1543, Xinluzao 26, and Xinluzhong 30 were germinated under controlled conditions and subjected to respective stress treatments.For instance, drought treatment involved the application of 15% PEG6000 to KK1543 and Xinluzao 26 at the two-leaf stage, while salt stress treatment was administered using 150 $mmol/L^{-1}$ NaCl to Xinluzao 26 and Xinluzhong 30. Cotton seedlings of Zhongzhimian 2 and Simian 3 were grown in soil pots, with subsequent

inoculation of Vd592 spores to the roots to induce infection.Root tissues from the treatments were sampled at various time points for RNA extraction and gene expression analysis. Additionally, RNA was extracted from stems of 06-146 and Xinhai14 post-inoculation with FOV to assess gene expression across different time intervals.

The study was conducted with three biological and technical replicates. Relative gene expression levels were calculated using the $2^{-\Delta\Delta t}$ method [68], with detailed primer information provided in Table S6.

### The determination of lignin content

The determination of total lignin content in cotton stems was referenced from Han et al. [69].

#### Abbreviations

| | |
|---|---|
| WGCNA | Weighted Gene Co-expression Network Analysis |
| VIGS | Virus-Induced Gene Sile-ncing |
| WGD | Whole-genome duplication |
| DPA | Days post-anthesis |
| LP | Lint percentage |
| TDZ | Thidiazuron |
| TDZ | Thidiazuron |
| *V. dahliae* | *Verticillium dahliae* |
| FOV | *Fusarium oxysporum f. sp. Vasinfectum* |
| F-PKM | Fragments per kilobase of exon model per million mapped |
| MeJA | Methylj-asmonat-e |
| hpi | Hours post inoculation |
| GTs | Glycosyltransferases |
| PAL | Phenylalanine Ammonia Lyase |
| 4CL | Coumarate CoA Ligase |
| PPO | Polyphenol Oxidase |
| PR1 | Pathogenesis-Related Protein 1 |
| AOC | Allene Oxide Cyclase |
| CHI | Chalcone Isomerase |
| SOD | Superoxide Dismutase |
| CAT | Catalase |
| ACO | Aconitase |
| HMM | Hidden Markov Model |
| PEG | Polyethylene glycol |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05584-6.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

#### Acknowledgements

All authors are grateful to the laboratory members for help, advice and discussion.

#### Author contributions

ZZQ, ZGL and JY designed the experiments and wrote the manuscript. ZZQ, ZGL and JY performed most of the experiments. ZZC assisted in the experiments, analyzed the data and discussed the results.

#### Funding

This work was funded by the National Key Laboratory of Cotton Bio-breeding and Integrated Utilization(CB2023A19),the Project of Innovation Team Building in Key Areas of Xinjiang Production and Construction Corps [XPCC] (2019CB008).

#### Data availability

All GhGT64 sequence information is available in the Cotton Functional Genomics Database (CottonFGD) (https://cottonfgd.org/about/download.html).

### Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare no competing interests.

Received: 22 March 2024 / Accepted: 11 September 2024
Published online: 30 September 2024

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## Publisher's note