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A Phi-Class Glutathione *S*-Transferase Gene for Verticillium Wilt Resistance in *Gossypium arboreum* Identified in a Genome-Wide Association Study

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Verticillium wilt disease is one of the most destructive biotic stresses faced by cotton plants. Here, we performed a genome-wide association study (GWAS) in 215 Chinese *Gossypium arboreum* accessions inoculated as seedlings with *Verticillium dahliae* to identify candidate loci involved in wilt resistance. We identified 309 loci that had a significant association with Verticillium wilt resistance and - log(*P*) values >5.0; the highest signal appeared on Ca3 in a 74 kb haplotype block. Five genes were also located within this haplotype block. One of these genes, *CG05*, was positioned close to the most significant SNP Ca3_23037225 (14 kb); expression of the gene was induced by *V. dahliae* or by treatment with salicylic acid (SA). Therefore, we suggest that *CG05* may respond to invasion by *V. dahliae* via an SA-related signaling pathway, and we designated this gene as *GaGSTF9*. We showed that *GaGSTF9* was a positive regulator of Verticillium wilt through the use of virus-induced gene silencing (VIGS) and overexpression in Arabidopsis. In addition, the glutathione *S*-transferase (GST) mutant *gstf9* of Arabidopsis was found to be more susceptible to Verticillium wilt than wild-type plants. The levels of endogenous SA and hydrogen peroxide had a significant effect on Arabidopsis plants that overexpressed *GaGSTF9*, indicating that GST may regulate reactive oxygen species content via catalytic reduction of the tripeptide glutathione (GSH), and then affect SA content. Our data demonstrated that *GaGSTF9* was a key regulator mediating cotton responses to *V. dahliae* and a potential candidate gene for cotton genetic improvement.

Keywords: Cotton; • *GaGSTF9* • Glutathione *S*-transferase • GWAS • Resistance gene • Salicylic acid.

Abbreviations: DI, disease index; d.p.i., days post-inoculation; ET, ethylene (ethephon); GWAS, genome-wide association study; GST, glutathione *S*-transferase; GSH, tripeptide glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; h.p.i. hours post-inculation; JA, jasmonic acid; MeJA, jasmonic acid methyl ester; NPR1, nonexpressor of pathogenesis-related (PR) genes 1; PR, pathogenesis related; qRT-PCR, quantitative real-time PCR; QTL, quantitative trait locus; RIL, recombinant inbred line; ROS, reactive oxygen sopecies; SA, salicylic acid; SNP, single nucleotide polymorphism; TRV, *Tobacco rattle virus* VIGS, virus-induced gene silencing; WT, wild type.

Introduction

Verticillium wilt is caused by a soil-borne fungus, *Verticillium dahliae*, and is one of the most destructive plant diseases worldwide. This fungus has been demonstrated to be strongly pathogenic in >200 plant species, including a wide range of high-value agricultural crops, such as cotton, lettuce, olive and potato (Klosterman et al. 2009, Inderbitzin and Subbarao 2014). Cotton, one of the most economically important crops in the world, has been increasingly affected by outbreaks of *V. dahliae*; in China, for example, the disease is present on most of the cotton acreage and results in huge economic losses every year (Mo et al. 2016, Zhang et al. 2016). Infection by *V. dahliae* results in the colonization of vascular tissues and causes malfunction of various metabolic processes to produce symptoms such as wilting, stunting, chlorosis, vascular browning discoloration and defoliation (Fradin and Thomma 2006, Jiménez-Díaz et al. 2012, Cheng et al. 2016, Santhanam et al. 2016). Although a wide range of chemical and biological treatments have been investigated as control agents for the disease, their effectiveness has been limited as *V. dahliae* can survive in the soil for many years without a host (López-Escudero and Mercado-Blanco 2010, Zhang et al. 2014).

Developing Verticillium wilt-resistant cultivars is an alternative approach to coping with the disease (Yu et al. 2017). *Gossypium arboreum* is one of four domesticated cotton species, and was first introduced into Southern China >2,000 years ago from India; it now forms the Chinese geographic race designated as *sinense* and provides a valuable gene pool for cultivating resistant cultivated species (Liu et al. 2005). Although *G. arboreum* is no longer the major cultivated species, having been replaced by upland cotton (*G. hirsutum*) since the 1950s, it is still an important germplasm resource for cotton breeding and has many accessions with diverse elite traits such as early maturity, tolerance to drought, good fiber quality and resistance to insects and disease (Guo et al. 2006). Artificial selection for desirable phenotypic characters has resulted in considerable

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genetic variation among accessions. Analyses of quantitative trait loci (QTLs) have been carried out to identify and characterize genes involved in important traits in experimental populations, such as F_2 progeny and recombinant inbred lines (RILs). However, QTL mapping faces two fundamental limitations: mapping resolution can be significantly restricted first by the frequency of recombination during creation of the RIL population, and secondly by a low level of genetic diversity segregating between the two parents. An alternative to QTL mapping is the use of genome-wide association studies (GWASs) that overcome the limitations of QTLs by use of a diverse population and high densities of single nucleotide polymorphism (SNP) markers to identify genes. GWAS has been proved to be a powerful tool for identification of key genes or loci regulating important traits through its ability to detect simultaneously large numbers of natural allelic variations underlying phenotypic variance in a single study (Korte and Farlow 2013). This analytical method has been used to identify regulators for key agronomic traits in rice (Huang et al. 2010), maize (Tian et al. 2011), tomato (Lin et al. 2014), soybean (Takagi et al. 2015) and sesame (Qi et al. 2014).

In this study, we used the GWAS approach to identify key regulators associated with Verticillium wilt resistance in *G. arboreum*. In total, 215 accessions, representing the whole range of genetic diversity in *G. arboreum* in China, were phenotyped for Verticillium wilt resistance in a greenhouse. In this GWAS, we sought to identify the causative genes or loci that regulate Verticillium wilt resistance as these will be invaluable for future breeding programs. A large number of significant loci were identified here, one of which, the Phi-class glutathione S-transferase *GaGSTF9*, was a candidate for Verticillium wilt resistance. We validated the function of *GaGSTF9* in cotton and Arabidopsis. We also examined the mechanism of action of *GaGSTF9* and found that it might participate in the regulation of *V. dahliae* infection via a salicylic acid (SA)-mediated signaling pathway.

Results

Phenotyping of Verticillium wilt resistance in natural population

In the current study, 215 cotton accessions were used as the association panel. These accessions were separated into three groups according to their geographic distributions: South China, Yangtze River and Yellow River (**Supplementary Table S1**). Verticillium wilt is highly sensitive to environmental and development factors, so a stable environment is necessary for its evaluation. Given the limitations of field evaluation, the present study employed an artificial inoculation method (Zhu et al. 2013) of plants grown in a greenhouse; three replicates of each treatment were analyzed (see the Materials and Methods). Assessment of the severity of Verticillium wilt showed that there was considerable variation in the disease index (DI). The DIs of the selected population ranged from 3.0 to 79.2, with an average of 21.80 (**Fig. 1A, C**). An analysis of variance (ANOVA) showed that DIs among different genotypes were significantly different ($F = 3.713$, $P < 0.0001$) (**Fig. 1B**).

Genome-wide association analysis

In our previous study of genetic variation in *G. arboreum*, we identified 1,568,133 high-quality SNPs from all lines. These SNPs provided the foundation for the GWAS performed here.

We carried out a trait–SNP association study using EMMAX (Kang et al. 2010) to identify loci involved in the genetic regulation of disease resistance. A quantile–quantile (QQ) plot showed that only the most significantly associated markers deviated from the expected *P*-values (**Fig. 1D**), which indicated that EMMAX could be used for identification of signals associated with disease. In total, 309 loci were identified with −log (*P*) values >5.0, and 22 loci with −log (*P*) values >6.0. Most of these loci were located on Ca3 (**Fig. 1E; Table 1**; **Supplementary Table S2**). Three peaks (Ca3, Ca5 and Ca7) were observed in the Manhattan plot, with the strongest and most continuous association on chromosome 3 (**Fig. 1E**). The SNP alleles at the three peaks were A/G, A/G and C/T, respectively. We compared the effects of allelic variation on Verticillium wilt resistance. At the first peak, the average DI of the 'A' allele was 20.06, which was much lower than that (41.06) of accessions carrying the 'G' allele ($P < 0.01$) (**Fig. 1F**). Similarly, the average DIs of accessions with the 'A' and 'C' alleles at Ca5_44181752 and Ca7_31237380 were both lower than the average DIs of accessions carrying the 'G' and 'T' alleles ($P < 0.01$) (**Fig. 1G, H**). Our results suggested that accessions carrying disease-susceptible genotypes were more sensitive to Verticillium wilt.

Prediction of candidate genes for Verticillium wilt resistance

A peak on Ca3 (Ca3_23037225) had the highest −log (*P*) value (7.45) and was designated SNP_{DI}. As this might be a major genetic locus for Verticillium wilt resistance in *G. arboreum* (**Fig. 2A**), we analyzed the haplotype block structure around Ca3_23037225 (~1 Mb on either side) to identify potential candidate genes. The analysis revealed that Ca3_23037225 was associated with a 74 kb haplotype block that contained 588 SNPs (**Fig. 2B**) and that five genes were located in this block (**Fig. 2C**). Gene ontology analysis showed that the five genes had been annotated as regulation of transcription, translation, RNA methylation, protein ubiquitination and defense response (**Table 2**).

To determine whether these genes were responsible for resistance to Verticillium wilt, we performed a quantitative real-time PCR (qRT-PCR) analysis to detect candidate gene expression in the resistant (R) genotype GA0099 and the susceptible (S) genotype GA0176 following *V. dahliae* treatment., Two genes (*CG02* and *CG05*) were up-regulated after *V. dahliae* inoculation, and both responded only to *V. dahliae* in the R genotype (**Fig. 2D, E**). The homologs of *CG02* and *CG05* in Arabidopsis encode a ribosomal protein L18ae/LX family protein and GST, respectively. GST has been reported previously to be involved in the disease resistance response in Arabidopsis (Perl-Treves et al. 2004, Sappl et al. 2004, Nutricati et al. 2006).

A recent study reported that *CG02* participates in resistance to Verticillium wilt in cotton through an SA-related pathway (Gong et al. 2017). However, as the possible role of *CG05* in Verticillium wilt resistance is currently unknown, we mainly

A

Enviroment	Mean	SD	Min	Max	CV(%)
Greenhouse	21.80	10.79	3.0	79.2	49.51

B

Source	df	SS	MS	F-value	P
Genotype	213	48641	228.36	3.713	<0.0001

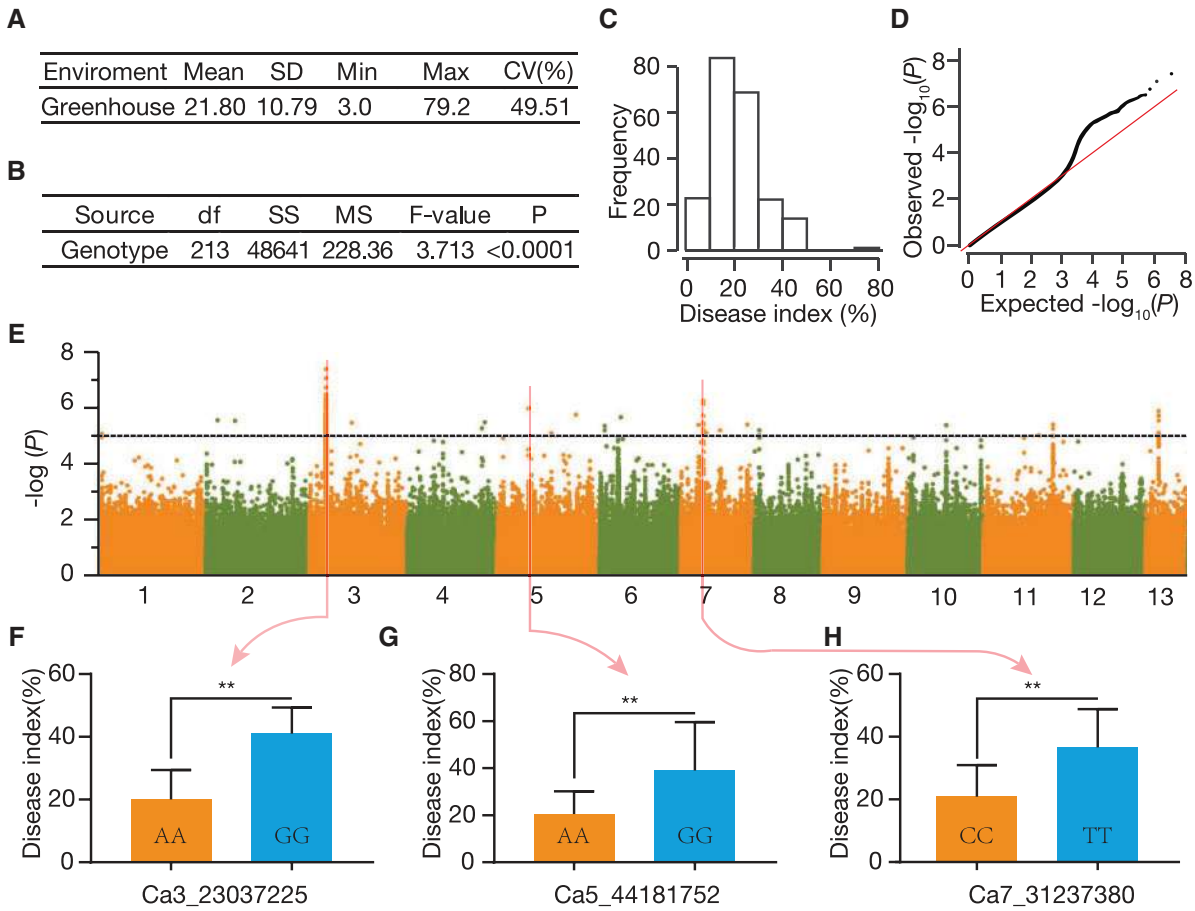

Fig. 1 Phenotypic variation and genome-wide association using SNPs and the disease index (DI). (A) Phenotypic variation. (B) Analysis of variance (ANOVA) results of Verticillium wilt resistance among different genotypes. (C) Frequency histogram of the DI: the *y*-axis shows the number of accessions with a DI in the range 0–10, 10–20, 20–30, 30–40, 40–50, 60–70 and 70–80. (D) Quantile–quantile plot for Verticillium wilt DI. (E) Manhattan plot for GWAS on the Verticillium wilt DI. (F, G, H) Relative DI of different alleles at the top three peaks (Student's *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$).

focused on this gene. We found that the first peak of the GWAS signal was closest to *CG05* (**Fig. 2F**). The average DIs of Haplotype A (Hap.A) and B (Hap.B) showed a statistically significant difference (**Fig. 1F**). The susceptible allele (shown in orange in **Fig. 2G**) was mainly carried by accessions from the South China region, and was not found in accessions from the Yangtze River/Yellow River region (**Fig. 2G**). This suggested that the agroecological environment has played a vital role in the development of Verticillium wilt resistance. The expression results showed that *CG05* was up-regulated in R genotype accessions (**Fig. 2H**), but not in S genotype lines (**Fig. 2I**). Our results therefore indicated that *CG05* was a potential candidate gene for the regulation of resistance to Verticillium wilt disease. Thus, we renamed *CG05* as *GaGSTF9* based on its homology with the Arabidopsis gene.

Expression of *GaGSTF9* in cotton under hormone treatment

In order to identify the signaling pathway associated with *GaGSTF9* expression, we analyzed expression patterns in the GA0099 accession following exogenous application of three different plant hormones [methyl jasmonate (MeJA), SA and ethephon (ET)]. We found that SA significantly induced expression of *GaGSTF9* at 6 h post-treatment; although MeJA and ET caused a slight increase in expression, these increases were not significant (**Fig. 2J**). Our results suggested that *GaGSTF9* was associated with Verticillium wilt resistance in cotton and that it responded to infection via an SA-related signaling pathway in Verticillium wilt-resistant accessions.

Silencing of *GaGSTF9* in cotton seriously compromises resistance to *V. dahliae* infection

In order to investigate further the function of *GaGSTF9* in cotton against *V. dahliae*, we used the *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) system to generate *GaGSTF9*-knockdown plants. To silence only the target gene, we designed specific primers for VIGS (**Supplementary Fig. S1**). Two weeks after *Agrobacterium* infiltration of GA0099, we used qRT-PCR to determine gene expression levels and estimated the relative silencing efficiency. The level of transcripts was obviously reduced in TRV::*GaGSTF9* plants compared with the control (**Fig. 3B**), suggesting that *GaGSTF9* was effectively silenced in TRV::*GaGSTF9* plants. No apparent symptoms of disease were observed in control

Table 1 SNPs associated with *Verticillium* wilt resistance

SNP	Position	SNP allele	MAF	$-\log_{10}(P)$
Ca3_22993830	22993830	G/T	0.056	7.12
Ca3_22994386	22994386	T/C	0.055	6.14
Ca3_22994401	22994401	T/C	0.058	6.52
Ca3_22994426	22994426	T/C	0.056	6.78
Ca3_22994649	22994649	T/C	0.058	6.38
Ca3_22999625	22999625	T/C	0.064	6.21
Ca3_23007250	23007250	A/C	0.063	6.02
Ca3_23011176	23011176	T/C	0.066	6.47
Ca3_23011189	23011189	G/A	0.067	6.33
Ca3_23011391	23011391	A/G	0.073	6.37
Ca3_23011732	23011732	G/A	0.065	6.10
Ca3_23014214	23014214	G/A	0.064	6.08
Ca3_23017000	23017000	T/A	0.071	6.01
Ca3_23019013	23019013	G/A	0.062	6.30
Ca3_23019036	23019036	T/C	0.062	6.30
Ca3_23024536	23024536	G/C	0.064	6.19
Ca3_23037225	23037225	A/G	0.060	7.45
Ca3_23038265	23038265	G/T	0.068	6.21
Ca3_23046761	23046761	T/C	0.067	6.53
Ca5_44181752	44181752	A/G	0.053	6.01
Ca7_31203570	31203570	C/T	0.060	6.17
Ca7_31237380	31237380	C/T	0.060	6.28

SNP allele, major and minor alleles; MAF, minor allele frequency.

Table 2 Candidate genes linked to the genomic region of the SNP most highly associated with *Verticillium* wilt resistance in cotton

Code	Gene	Start	Stop	Arabidopsis homologs	Description	GO annotation
CG01	Cotton_A_12317	22985258	22987176	AT4G35540.1	Zinc ion binding; transcription regulators	Regulation of transcription
CG02	Cotton_A_12318	22987558	22988768	AT2G34480.1	Ribosomal protein L18ae/LX family protein	Translation
CG03	Cotton_A_12319	22994945	23007225	AT1G06560.1	NOL1/NOP2/sun family protein	RNA methylation
CG04	Cotton_A_12320	23035835	23023765	AT1G06590.1	Anaphase-promoting complex subunit	Protein ubiquitination
CG05	Cotton_A_12321	23049529	23051289	AT2G30860.2	Glutathione S-transferase Phi 9	Defense response

TRV::00 plant tissues; in contrast, necrotic, yellowish, stunted and wilting leaves were found in TRV::*GaGSTF9* plants after inoculation with *V. dahliae* (**Fig. 3A**). The DI values for TRV::*GaGSTF9* plants were significantly higher than those of the control at 20 and 25 days post-inoculation (d.p.i.) (**Fig. 3C**). Following the method described in a previous report (Atallah et al. 2007), we used qRT-PCR to detect and quantify *V. dahliae* colonization. The expression levels of *V. dahliae* in TRV::*GaGSTF9* plants were higher than in TRV::00 plants, demonstrating that the former was sensitive to *V. dahliae* (**Fig. 3D**). To determine whether compromised plant resistance to *V. dahliae* was associated with *GaGSTF9* silencing, we used a recovery assay to examine the degree of colonization of *V. dahliae* in the infected stems of treated plants. By counting stem sections showing fungal growth, we can estimate the level of harmful fungus on the plants. The analysis showed that significantly more fungal colonies were present on TRV::*GaGSTF9* plants than on control plants (**Fig. 3E**). Examination of cotton cell morphology after pathogen inoculation showed that cells in TRV::*GaGSTF9* plant vascular bundles were longer and larger than in the control, and that the mycelium of *V. dahliae* appeared to penetrate more easily into TRV::*GaGSTF9* plants than the control plants (**Fig. 3F**). To compare the differences in the leaves of plants inoculated with *V. dahliae*, we used trypan blue to stain dead cells. The trypan blue staining area was larger and the color was more intense in the TRV::*GaGSTF9* leaves, and particularly in the veins, than in the control leaves (**Fig. 3G**). In summary, our results confirmed that *GaGSTF9* knock-down changed a resistant accession into a susceptible one, and that *GaGSTF9* may work as a positive regulator for Verticillium wilt resistance in cotton.

Transgenic Arabidopsis seeds are resistant to *V. dahliae*

Arabidopsis thaliana plants of the Columbia ecotype (Col-0) were transformed to generate *GaGSTF9* overexpression lines

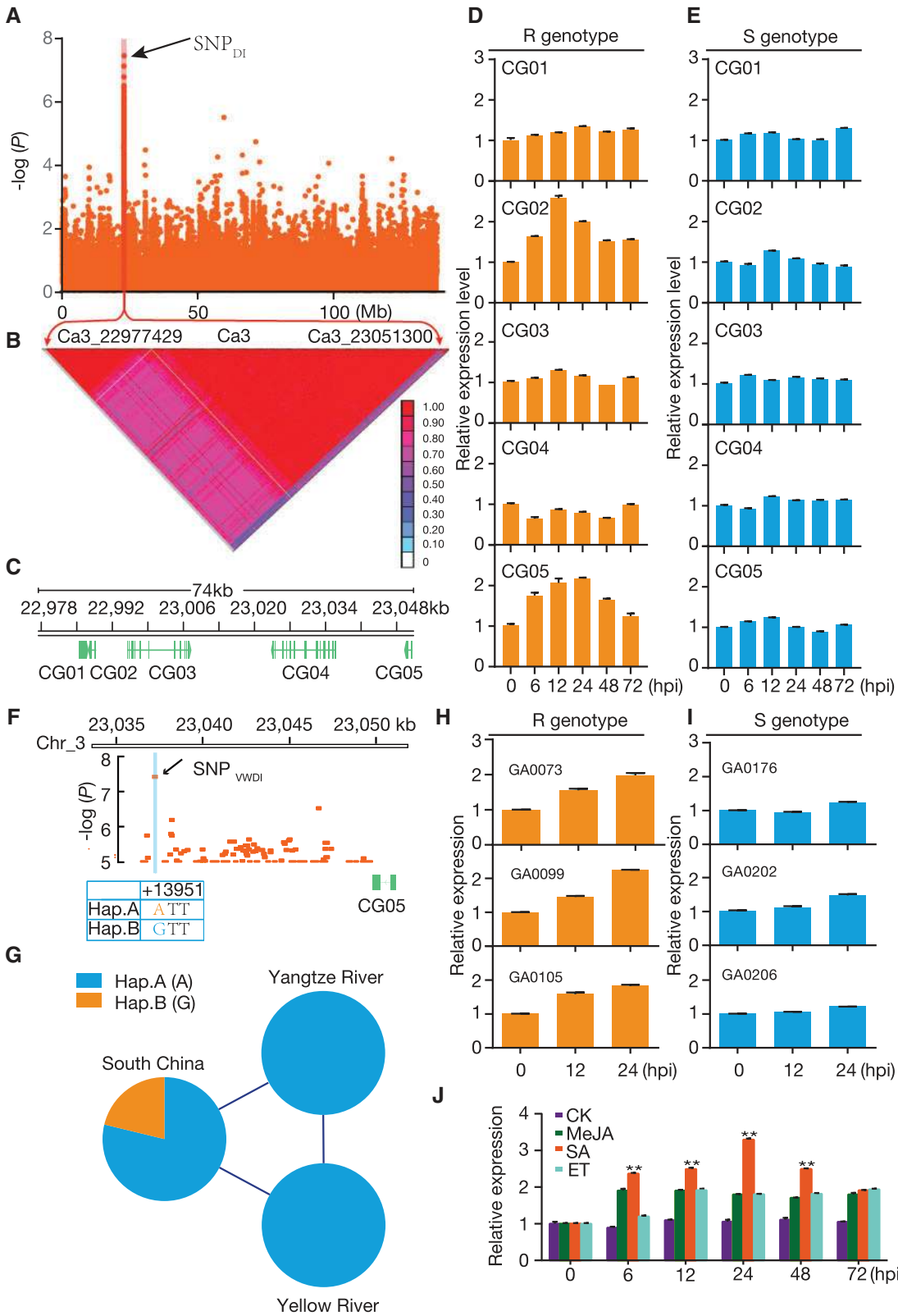

Fig. 2 Identification of a key regulator, *GaGSTF9*, against Verticillium wilt disease in a GWAS and its expression patterns under hormone treatment in cotton. (A) Manhattan plot for Verticillium wilt on chromosome Ca3. (B) Haplotype block around the peak. (C) Five candidate genes are located in the haplotype block. (D, E) Expression patterns of the candidate genes at 0, 6, 12, 24, 48 and 72 h.p.i. with *V. dahliae* strain V07038 in GA0099 (R genotype) and GA0176 (S genotype). (F) Enlarged view of the strongest signal in (A). (G) Frequencies of Hap.A and Hap.B in three geographical regions in China. (H, I) Relative expression of *GaGSTF9* in roots of the resistant lines GA0073, GA0099 and GA0105 (R genotype) and the susceptible lines GA0176, GA0202 and GA0206 (S genotype) at 0, 12 and 24 h post-*V. dahliae* inoculation (the error bar

driven by the 35S promoter. We used various methods to select homozygous *GaGSTF9* transgenic lines, including Basta spray (0.1%), PCR, qRT-PCR and Southern blot (**Supplementary Fig. S2**). When we obtained homozygous overexpression lines in the T_4 generation, we tested disease resistance using an in vitro technique. Comparisons were made daily between *GaGSTF9* transgenic plants and WT plants. As expected, the transgenic Arabidopsis plants exhibited enhanced resistance to *V. dahliae* compared with WT plants; the WT plants were on the verge of death after 14 d treatment with *V. dahliae*, while the transgenic plants were still healthy (**Fig. 4A; Supplementary Fig. S3A**). In WT plants, wilt symptoms occurred as early as 6 d.p.i., and the plants rapidly become yellow with stunted growth. Counting the numbers of stunted and chlorotic leaves in transgenic and WT plants at 14 d.p.i. gave an estimate of stunting in WT plants of 3 compared with 2 in transgenic plants (**Fig. 4B; Supplementary Fig. S3B**); the extent of chlorosis in WT plants was 4, while in transgenic plants it was 2 or 3 (**Fig. 4C; Supplementary Fig. S3C**). Our results confirmed that transgenic Arabidopsis is more resistant to *V. dahliae* infection than WT plants.

Effects of overexpression of *GaGSTF9* on SA signalng pathways in Arabidopsis under *V. dahliae* challenge

GaGSTF9 is associated with the SA signaling pathway in cotton; however, it is uncertain whether overexpression of *GaGSTF9* affected the SA signaling pathways in Arabidopsis. To determine the effects of *GaGSTF9* overexpression, we inoculated transgenic *A. thaliana* with *V. dahliae* and then screened expression levels of marker genes in the SA signal pathways, including the biosynthesis of SA (*AtNPR1*, *AtPR1* and *AtPR*3). As shown in **Fig. 4D** and **Supplementary Fig. S3D**, the expression levels of SA-related genes in the background under uninduced conditions were significantly changed in transgenic *Arabidopsis* plants compared with WT plants, and the expression levels increasing rapidly at 24 hours post-inoculation (h.p.i.) to a greater extent in transgenic plants than in WT plants. Therefore, overexpression of *GaGSTF9* had a clear influence on SA biosynthesis pathway genes. These results indicate that *GaGSTF9* may be involved in the SA biosynthesis pathway and thereby participate in Verticillium wilt defense.

Altered SA, GST and H_2O_2 levels after *V. dahliae* infection of plants overexpressing *GaGSTF9*

To evaluate further the function of *GaGSTF9* and confirm the results of the marker gene expression analysis in Arabidopsis, we analyzed the levels of SA, activity of GST and immune-response production of hydrogen peroxide (H_2O_2). Consistent with our analysis of expression of SA biosynthesis pathway marker genes (**Fig. 4D; Supplementary Fig. S3D**), the background level of endogenous SA was lower in transgenic Arabidopsis than in the WT under normal growth conditions; however, SA content increased more in transgenic (OE4, OE5 and OE13) than in WT plants after treatment with *V. dahliae* (**Fig. 4E; Supplementary Fig. S3E**). Overexpression of GaGSTF9 enhanced GST activity in transgenic plants, and GST activity in these plants showed a greater response to *V. dahliae* infection than in WT plants (**Fig. 4F; Supplementary Fig. S3F**). In addition, the endogenous H_2O_2 level was significantly lower in transgenic plants than in WT plants under normal growth conditions. The level of H_2O_2 rose dramatically in WT plants, but only increased slightly in transgenic plants after treatment with *V. dahliae* (**Fig. 4G; Supplementary Fig. S3G**). Our data showed that the increment of GST activity in transgenic plants effectively removed excess reactive oxygen species (ROS) and maintained a low level of H_2O_2 to protect the plants; the SA signaling pathway was involved in these responses.

Exogenous application of SA makes transgenic Arabidopsis more resistant to *V. dahliae*

Next, we further characterized the function of *GaGSTF9* by analyzing resistance of Arabidopsis *GaGSTF9* transgenic plants to treatment with *V. dahliae*. Following inoculation of T_4 lines with *V. dahliae*, *GaGSTF9* transgenic plants showed enhanced resistance compared with that of WT plants (**Fig. 5A; Supplementary Fig. S4A**). We also pre-treated transgenic Arabidopsis and WT seedlings at the rosette leaf stage with 1 mM SA for 24 h, inoculated the seedlings with *V. dahliae* and gave them an exogenous supplement of SA each day for 4 d. We found that exogenous SA significantly increased resistance to pathogen stress in transgenic Arabidopsis plants (**Fig. 5B; Supplementary Fig. S4B**). Comparison of rosette leaves after infection showed that SA enhanced resistance to *V. dahliae* in transgenic Arabidopsis plants but had little impact on WT plants (**Fig. 5C; Supplementary Fig. S4C**). Estimation of the DI and relative amount of *V. dahliae* DNA in WT plants, SA-treated WT plants, transgenic lines and SA-treated transgenic lines showed that WT plants had the highest DI and highest fungal DNA level, followed by WT plants treated with SA, and then transgenic and transgenic plants treated with SA (**Fig. 5D, E; Supplementary Fig. S4E**, **F**). At 10 d.p.i., the DIs of control transgenic plants and those plants treated with SA were not significantly different by a one-way ANOVA (**Fig. 5D**), indicating that the transgenic plants had enhanced resistance to *V. dahliae*, which delayed the onset of the disease. However, at 15 d.p.i., SA treatment produced significantly better resistance to *V. dahliae* in transgenic plants than in WT plants.

Fig. 2 Continued
indicates the SD, $n = 3$). (J) Three-week-old seedlings of the resistant accession GA0099 were treated with exogenous hormones or an equal amount of sterile distilled water. Mock and treated leaves were harvested at 0, 6, 12, 24, 48 and 72 h.p.i., and gene expression levels were determined by qRT-PCR using *his*3 as the internal reference gene. The expression level of the Mock control was normalized as '1'; error bars represent the SD of three biological replicates. Asterisks indicate statistically significant differences, as determined by Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

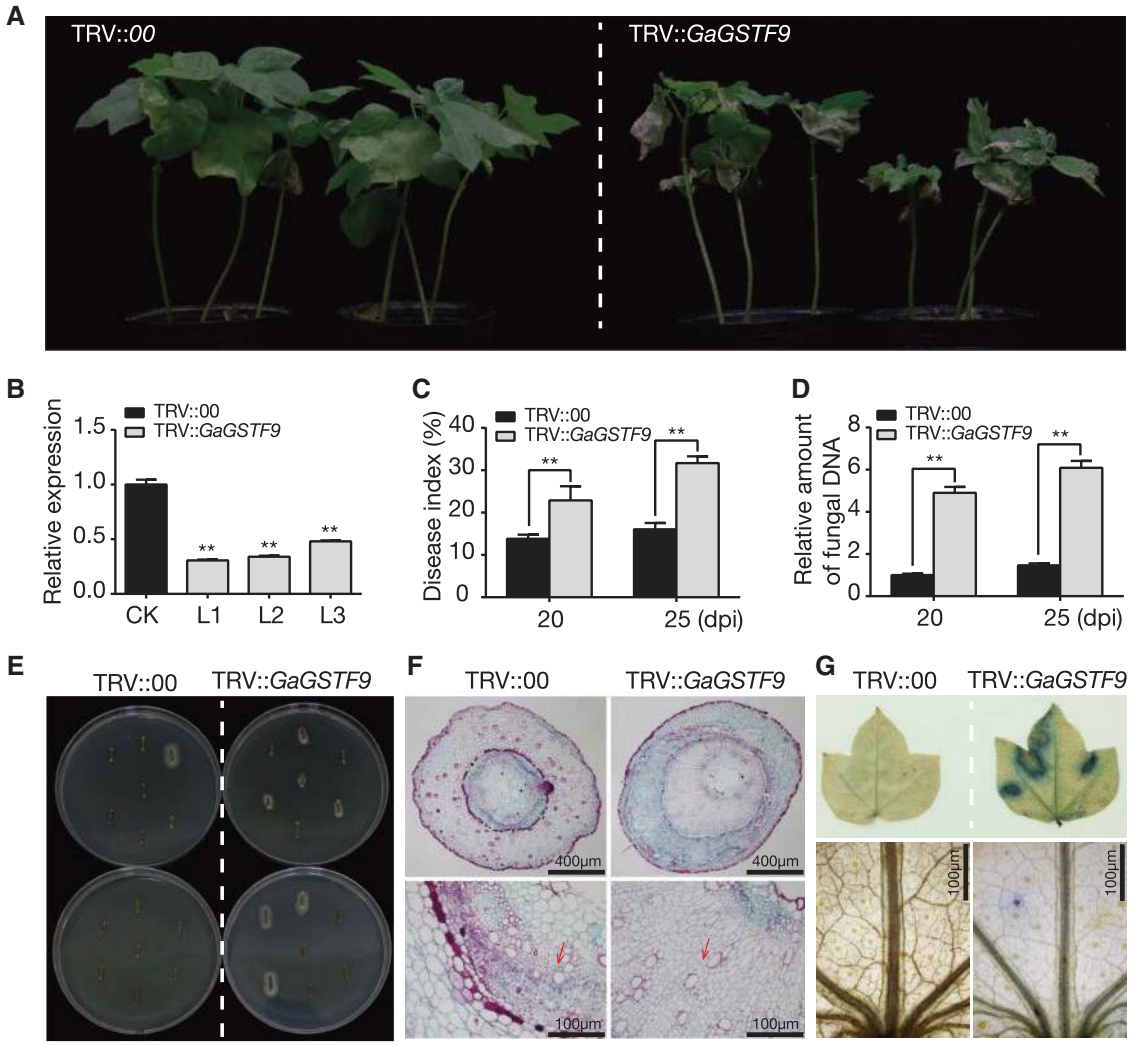

Fig. 3 Silencing of *GaGSTF9* in cotton compromises *V. dahliae* resistance. (A) Disease symptoms induced by *V. dahliae* on the leaves of TRV::00 and TRV::*GaGSTF9* plants at 25 d.p.i. (B) Transcript levels of *GaGSTF9* in leaves of TRV::00 and TRV::*GaGSTF9* plants; the levels were determined by qRT-PCR using *his3* as the internal reference gene. The value of TRV::00 was normalized to '1'. (C) Assessment of the DI at 20 and 25 d.p.i. Error bars represent the SD of three biological replicates ($n \geq 30$). (D) Quantification of *V. dahliae* biomass by qRT-PCR on the leaves of TRV::00 and TRV::*GaGSTF9* plants at 20 and 25 d.p.i. Error bars represent the SD of three biological replicates. Asterisks indicate statistically significant differences, as determined by Student's *t*-test (*$P < 0.05$; **$P < 0.01$). (E) Stem sections (25 d.p.i.) were plated on potato dextrose agar medium and incubated at 25 °C. Photographs were taken at 7 d after plating. (F) Cell morphology in stems of TRV::00 and TRV::*GaGSTF9* cotton plants at 25 d.p.i. (G) Trypan blue staining of *V. dahliae*-infected leaves of TRV::00 and TRV::*GaGSTF9* cotton plants at 25 d.p.i.

The relative amount of fungal DNA in SA-treated plants was lower and the SA treatment appeared to be more beneficial against *V. dahliae* infection in the overexpression plants. The difference in relative DNA content was greatest between control and SA-treated transgenic plants, but that between WT and SA-treated WT plants was only significant at 15 d.p.i.

We also used a recovery assay to examine the degree of colonization of *V. dahliae* in infected stems and found that the number of colonies was significantly different between WT control, transgenic lines and SA-treated transgenic lines, while SA-treated WT lines had the same level as WT plants (**Fig. 5F; Supplementary Fig. S4D**). Thus, we confirmed that *GaGSTF9* was associated with SA signaling pathways, which enhanced resistance to *V. dahliae* in transgenic Arabidopsis.

Loss of *GSTF9* gene expression in Arabidopsis makes plants more susceptible to *V. dahliae*

In order to assess whether *GSTF9* has a role in resistance to *V. dahliae*, we examined an Arabidopsis mutant with a knockout of *GSTF9* (At2g30860); this mutation is identified as Salk 001519C in the SALK database and was obtained from the Arabidopsis Biological Resource Center. Plants homozygous for the mutation were selected by PCR and infected with *V. dahliae*. As expected, the *gstf9* mutant was more susceptible to *V. dahliae* than WT plants. After inoculation with *V. dahliae*, yellowish, stunted and wilting leaves were observed in WT plants, whereas *gstf9* mutant plants were completely wilted, yellow and almost dead (**Fig. 6A**). We compared rosette leaves from WT plants and *gstf9* mutant

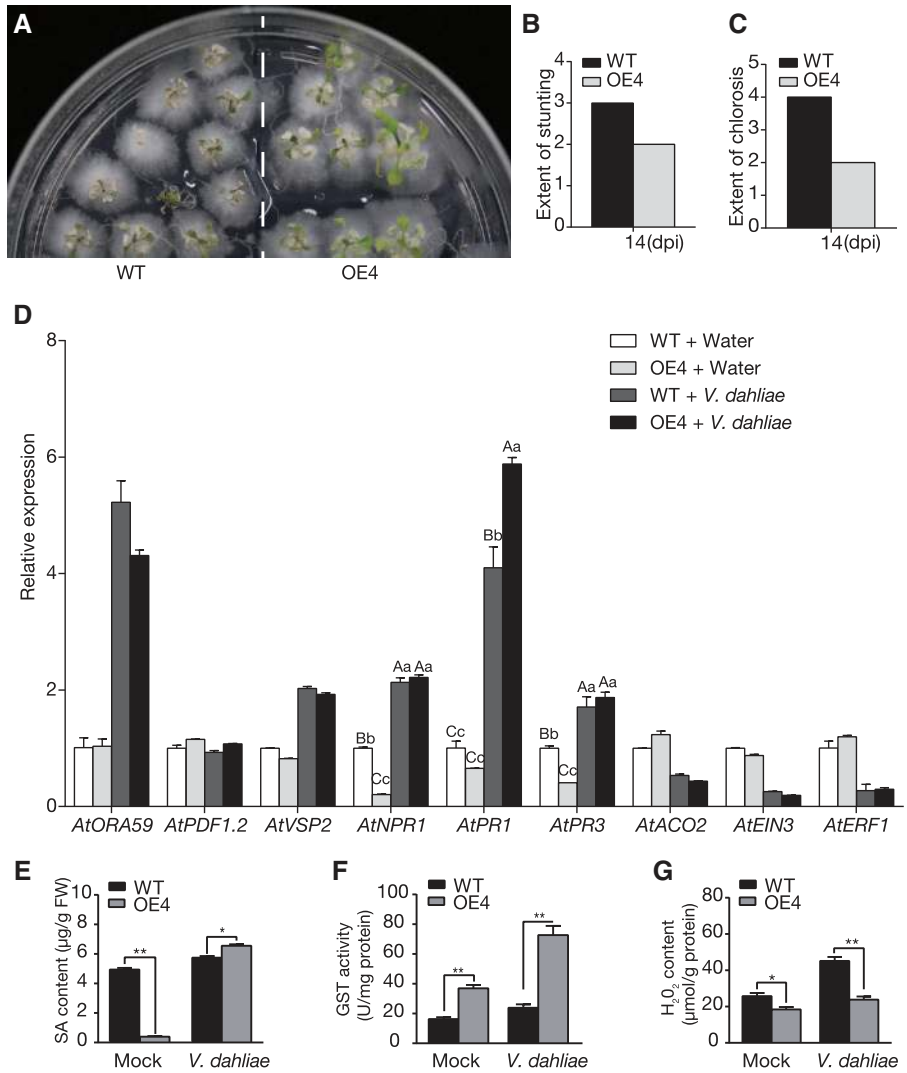

Fig. 4 Transgenic Arabidopsis seedlings are more resistant to Verticillium wilt and the effects of overexpression of *GaGSTF9*. (A) Disease symptoms in in vitro grown seedlings. Two-week-old plantlets were treated with *V. dahliae*. Photographs were taken at 14 d.p.i. (B) Extent of stunting in WT and 35S::*GaGSTF9* plants at 14 d.p.i. (C) Extent of leaf chlorosis in WT and 35S::*GaGSTF9* plants at 14 d.p.i. (D) Expression patterns of SA signaling pathway-related marker genes in WT and *GaGSTF9* transgenic Arabidopsis after *V. dahliae* infection for 24 h. The expression levels were assessed by qRT-PCR using *AtUBQ10* as the internal control gene. One-way ANOVA was used in the statistical analysis of the data, and results were confirmed using a Duncan's multiple range test. Lower and upper case represent comparisons at $\alpha = 0.05$ and $\alpha = 0.01$ levels, respectively. Error bars represent the SD of three biological replicates. (E) HPLC measurement of leaf SA in WT and 35S::*GaGSTF9* plants at 0 and 24 h.p.i. Bars represent SEs from three biological replicates. Asterisks indicate statistically significant differences (Student's *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$). (F, G) Leaves of WT and 35S::*GaGSTF9* plants were harvested at 0 and 24 h.p.i. to measure the activity of GST and the H_2O_2 content. Error bars represent the SD of three biological replicates. Asterisks indicate statistically significant differences when compared with control (Student's *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$).

plants at 10 d.p.i. (**Fig. 6B**), calculated DI values and quantified the *V. dahliae* colonization using qRT-PCR. The DI value for *gstf9* mutant plants was significantly higher than that of WT plants (**Fig. 6C**). Analyses of the expression level of fungal genes revealed that WT plants were more resistant to *V. dahliae* than *gstf9* mutant plants (**Fig. 6D**). We also analyzed the levels of SA and found that the background level of endogenous SA was lower in WT plants than in *gstf9* mutant plants under normal growth conditions. SA contents in *gstf9* mutant plants and WT plants were approximately the same after treatment with *V. dahliae* (**Fig. 6E**). Though the contents of SA in the *gstf9* mutant and WT were consistent at 24 h.p.i., the lack of *GSTF9* reduced the ability to remove cytotoxic molecules and ROS, causing *gstf9* mutant plants to be more susceptible than WT plants.

Discussion

GaGSTF9 is a candidate gene for wilt resistance

The natural variation in *G. arboreum* to Verticillium wilt was investigated using GWAS to identify candidate genes and loci regulating the response. In total, 215 *G. arboreum* accessions,

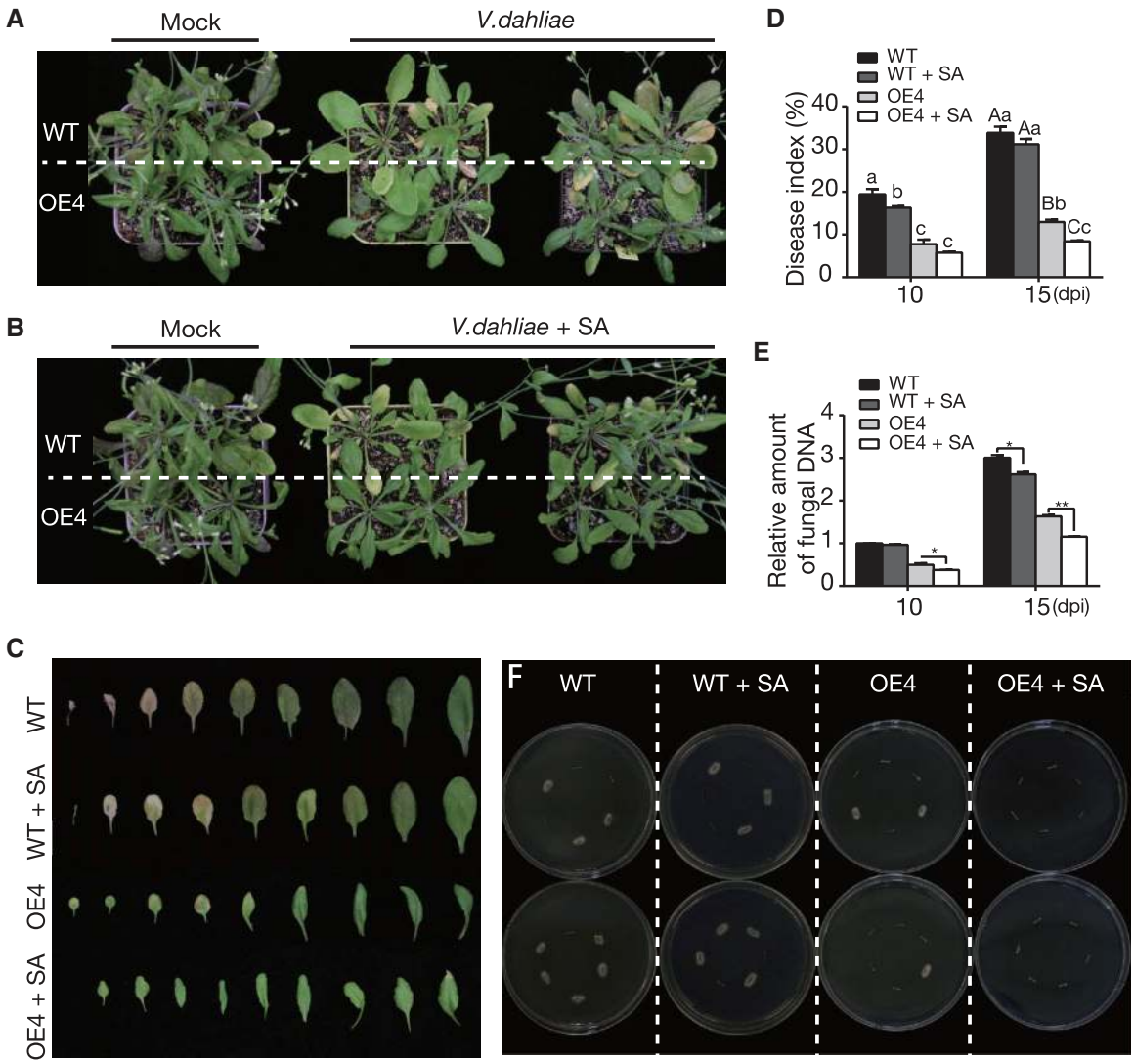

Fig. 5 Effects of overexpression of *GaGSTF9* and exogenous SA in defense responses to *Verticillium dahliae* in Arabidopsis. (A, B) Disease symptoms induced by *V. dahliae* on the rosette leaves of WT, *GaGSTF9*-overexpressing plants and exogenous SA-treated plants. Three-week-old seedlings were inoculated with *V. dahliae*, and transgenic Arabidopsis and WT seedling rosette leaves were pre-treated with 1 mM SA for 24 h before inoculation with *V. dahliae*. Exogenous SA was applied every day for 4 d. Control plants were sprayed with water. Photographs were taken at 15 d.p.i. (C) Appearance of Arabidopsis rosette leaves. (D) Assessment of the DI at 10 and 15 d.p.i. Error bars represent the SD of three biological replicates ($n \geq 30$). One-way ANOVA was used in statistical analysis of the data, and the results were confirmed using a Duncan's multiple range test. Lower and upper case represent comparisons at $\alpha = 0.05$ and $\alpha = 0.01$ levels., respectively. (E) Quantification of the *V. dahliae* biomass by qRT-PCR using *AtUBQ10* as the internal control gene in rosette leaves of WT, *GaGSTF9*-overexpressing and SA-treated plants at 10 and 15 d.p.i. Error bars represent the SD of three biological replicates. Asterisks indicate statistically significant differences, as determined by Student's *t*-test (*$P < 0.05$; **$P < 0.01$). (F) Stem sections (15 d.p.i.) were plated on potato dextrose agar medium and incubated at 25 °C. Photographs were taken at 7 d after plating.

which represent the range of genetic diversity in China, were analyzed. Next-generation sequencing of the panel of 215 accessions provided a high-density SNP marker map, which had higher resolution than that previously developed using QTLs (Yang et al. 2008); the increased resolution makes mapping of genes regulating phenotypic traits more convenient and efficient. Our study using GWAS identified *GaGSTF9* as a candidate for response to *V. dahliae* infection. This approach has also been exploited to identify genes for important agronomic traits in other crop species; for example, in tomato, *SlMYB12* was shown to regulate the development of pink fruit (Lin et al. 2014); in sesame, *SiPPO* was identified as a candidate gene for oil content (Wei et al. 2015); in rice, *NAL1* was shown simultaneously to regulate panicle number per plant, spikelet number per panicle and leaf blade length (Yano et al. 2016); and, in maize, *LIGULELESS* was found to contribute to the development of more upright leaves (Tian et al. 2011). *GaGSTF9* is a member of the Phi class of GST genes and is a novel gene for Verticillium wilt resistance in cotton. Although GSTs have been thoroughly studied in *A. thaliana* (Hayes et al. 2005), very little information

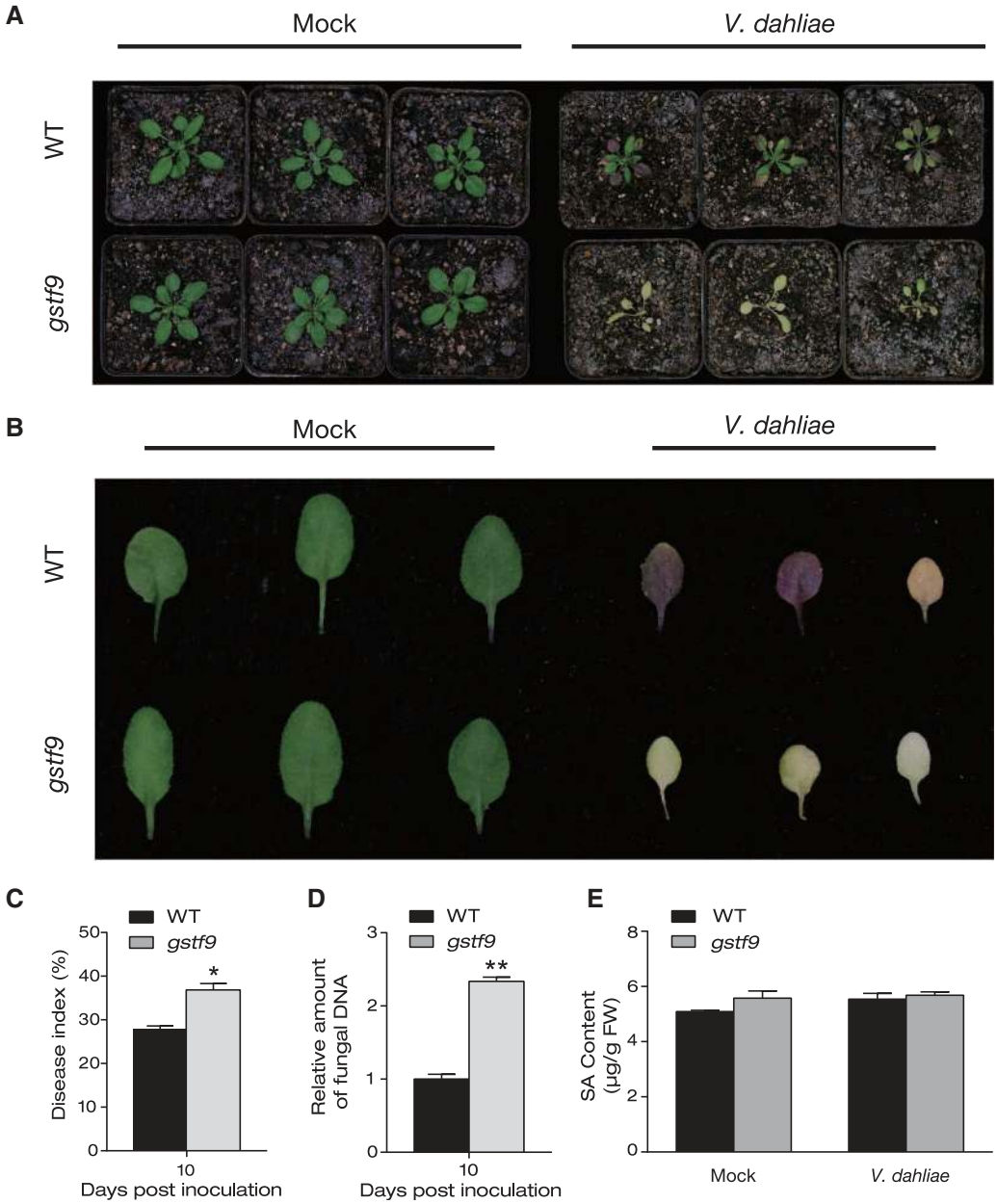

Fig. 6 The Arabidopsis *gstf9* mutant is more susceptible to Verticillium wilt than the WT. (A) Disease symptoms induced by *V. dahliae* on the rosette leaves of WT and *gstf9* mutant plants. Three-week-old seedlings were inoculated with *V. dahliae*, and control plants were sprayed with water. Photographs were taken at 10 d.p.i. (B) Appearance of Arabidopsis rosette leaves. (C) Assessment of the DI at 10 d.p.i. Error bars represent the SD of three biological replicates ($n \geq 30$). Asterisks indicate statistically significant differences as determined by Student's *t*-test (*$P < 0.05$). (D) Quantification of the *V. dahliae* biomass by qRT-PCR using *AtUBQ10* as the internal control gene in rosette leaves of WT and *gstf9* mutant plants at 10 d.p.i. Error bars represent the SD of three biological replicates. Asterisks indicate statistically significant differences as determined by Student's *t*-test (**$P < 0.01$). (E) HPLC measurements of leaf SA in WT and *gstf9* mutant plants at 0 and 24 h.p.i. Bars represent SEs from three biological replicates. Asterisks indicate statistically significant differences (Student's *t*-test; *$P < 0.05$; **$P < 0.01$).

is available on the relationship between Phi class proteins and Verticillium wilt. Moreover, the functions of GSTs are largely unknown in cotton.

Gossypium arboreum was first introduced into South China; thus accessions from this region are more ancient than those from the Yangtze River and Yellow River (Liu et al. 2005). The allelotypes of SNP_{DI} in 15 accessions of *G. herbaceum* ($2n = 26$, A_1 genome), a close relative of *G. arboretum*, and of upland cotton TM-1 [$2n = 4x = 52$, $(AD)_1$ genome] are all Hap.B (data not shown). We therefore speculate that Hap.B at SNP_{DI}, which is carried by most accessions from South China, is the ancient genotype. Accessions carrying Hap.B were more susceptible to *V. dahliae*; the disappearance of Hap.B from Yangtze River and Yellow River accessions suggests that geographic selection played an important role in Verticillium wilt resistance in *G. arboreum*.

Silencing of *GaGSTF9* in cotton compromises resistance to *V. dahliae* infection

Studies on the GST protein family have shown that these proteins have roles in direct cytoprotective activities and that they might be essential to protect plants against environmental stresses and a variety of diseases (Edwards et al. 2000). Some GSTs have been studied in Arabidopsis and their functions in plant defense systems have been investigated. For example, the defense-related gene *AtGSTF6* shows significant up-regulation in Arabidopsis exposed to the fungal pathogen *Erysiphe orontii* (Reuber and Wood 1998). In a similar manner, transcription of *AtGSTU26* is specifically induced by an attack by *Pseudomonas syringae* (Nutricati et al. 2006). Here, we hypothesized that *GaGSTF9* has a role in resistance to *V. dahliae*. The use of VIGS to block expression of *GaGSTF9* in resistant cotton caused the plants to become more susceptible to *V. dahliae* infection than control plants. This result is consistent with previous studies and confirms our hypothesis. To extend our observations, we assessed a series of resistance parameters, including DI, *V. dahliae* colonization, recovery assays and trypan blue staining. Silencing of *GaGSTF9* increased all four end-points in transgenic plants compared with vector control plants. Our results demonstrate that *GaGSTF9* is important in the process of resistance against *V. dahliae*.

Overexpression of *GaGSTF9* in Arabidopsis improves resistance to *V. dahliae* and affects endogenous SA levels

Although the association between GSTs and fungal invasion has been reported, most of these studies only examined the level of induced expression. In contrast, in the present study, we confirmed our conclusions on the function of *GaGSTF9* by generating transgenic Arabidopsis plants and examining their resistance to Verticillium wilt. *GaGSTF9*-overexpressing Arabidopsis plants showed more resistance to *V. dahliae* than the WT. Our results showed that overexpression of *GaGSTF9* enhanced resistance to *V. dahliae* in Arabidopsis seedlings and confirmed that *GaGSTF9* is an excellent candidate as a resistance gene. In the wild, plants frequently come into contact with a broad range of potential pathogens and, as a result, have evolved mechanisms to cope with these threats. Thus, endogenous signaling molecules, such as JA, SA and ET, participate in regulating plant defense responses (Balbi and Devoto 2008, Bu et al. 2014). The plant hormone SA plays an important role in disease resistance responses and can stimulate the expression of a series of disease resistance genes, such as non-expressor of pathogenesis-related genes (NPR1), pathogenesis-related genes (PR1) and WRKY transcription factor genes (Pieterse et al. 2012). In addition, GSTs are also induced by SA, and the relationship between them is inseparable. It has been suggested that the expression of *AtGSTU19* is induced by SA (Meyer et al. 2012). Analyses of GSTs have shown that they appear to display class-specific dependent SA induction, with significantly increased expression in both Phi and tau classes in Arabidopsis. Different members of the same class share similar SA dose responses (Sappl et al. 2004). However, these studies all examined the effects of SA on GSTs, but the effects of GSTs on SA have not been reported. Here, the levels of transcripts of SA-related marker genes indicated that the level of endogenous SA in transgenic plants was lower than in WT plants, and SA assays confirmed this conclusion. Our data indicated that excess expression of *GaGSTF9* in transgenic Arabidopsis affected the level of endogenous SA. It is worth noting that endogenous SA rose rapidly in transgenic plants to a level even higher than in WT plants at 24 h.p.i. These results suggested that *GaGSTF9* affects endogenous SA but had no effect on defense mechanisms in plants. In addition, our study also showed that exogenous SA enhanced the resistance to *V. dahliae* in transgenic Arabidopsis plants, but had little impact on WT plants. Furthermore, loss of *GSTF9* in Arabidopsis resulted in the level of endogenous SA being higher than in WT plants. The combination of all these results indicates not only that SA induces GSTs, but also that GSTs can affect SA by a complex reverse process; overall, GSTs and SA work together to resist the invasion of pathogens.

GSTs play an important role in resistance to various stresses

GSTs catalyze the conjugation of glutathione (GSH) to a wide variety of electrophilic substrates, including xenobiotic pollutants and endogenous metabolites. GSTs also have other functions, notably as glutathione peroxidases that catalyze GSH binding with ROS (Dixon et al. 2009). ROS are generated when plants are subjected to various stresses. One study indicated that enhancement of the ROS-scavenging systems in plants can provide partial protection from oxidative damage, thereby improving the stress tolerance of the plants (Roxas 2000). It has also been shown that AmGSTF2, activated as a glutathione peroxidase, was capable of significantly reducing organic hydro- peroxides in blackgrass (*Alopecurus myosuroides*) (Cummins et al. 1999). Glutathione peroxidases can catalyze the reaction of GSH with ROS to form oxidized glutathione (GSSG) (Wirth 2015). Bibi et al. (2013) reported that overexpression of the *GST* gene significantly reduces the accumulation of ROS. These various results are consistent with our analyses here and with our conclusion that *GaGSTF9* is involved in a reaction with ROS.

Accumulation of ROS triggers SA biosynthesis, which is an essential part of the disease resistance response in plants (Chaouch et al. 2010). However, SA can also promote ROS production. The combination of both processes produces a balance in cross-talk between SA and ROS (Khokon et al. 2011, Herrera-Vasquez et al. 2015). It has been shown that SA and GSH interact to regulate redox reactions; in this process, SA plays a critical role by regulating both GSTs and GSH (Chen and Singh 1999, Mateo et al. 2006). The overaccumulation of SA increases the levels of GSH in plants (Mateo et al. 2006), whereas the *catalase2* mutant of Arabidopsis abolishes the accumulation of SA, and GSH decreases compared with the WT (Noctor et al. 2015). In addition, the phytoalexin-deficient (*pad2-1*) mutant of Arabidopsis, which is deficient in GSH biosynthesis, has a lower level of SA (Dubreuil-Maurizi et al. 2011). The behavior of these mutants indicates that although SA can

induce the production of GSTs, it also promotes the conversion of GSSG to GSH. As previously mentioned in this study, many GSTs can be induced by SA, while overexpression of *GaGSTF9* reduced the accumulation of SA; the *gstf9* mutant of Arabidopsis showed an increased accumulation of SA. Thus, ROS and SA are connected by GSSG and GSH to form a regulatory loop. In this loop, GSTs are induced not only by SA, but also through the role of GSH, and then affects ROS and SA. Taken together, we propose that higher GST levels increase the consumption of ROS and cytotoxic molecules, thereby reducing the amount of ROS to a low level and affecting SA levels in 35S::*GaGSTF9* plants. When plants are exposed to stress, the amount of ROS rapidly rises, promoting an increase in SA and inducing defense responses. In WT plants, ROS and cytotoxic molecules are scavenged slowly due to a low background level of GSTs. In contrast, the increased GST transcripts in transgenic plants lead to an increased ability to catalyze GSH binding with ROS and cytotoxic molecules, thereby improving the stress tolerance of plants. In *gstf9* mutant plants, lower GST levels reduce the consumption of ROS and cytotoxic molecules, and affect the levels of ROS and SA. When plants are treated with *V. dahliae*, ROS and cytotoxic molecules are cleared more rapidlly in WT plants than in *gstf9* mutant plants, which makes the WT plants more resistant.

In summary, GWAS was used to analyze natural variation in 215 accessions for resistance to Verticillium wilt. A large of number of candidate loci were detected; among these was *GaGSTF9*, a Phi class member of the GST protein family, which was identified as a causative gene located in the strongest signal region in a Manhattan plot. Experiments in cotton and *A. thaliana* proved that *GaGSTF9* plays an important role in resistance to Verticillium wilt. In addition, our study provided data of value for understanding the mechanism by which GSTs aid resistance to invasion of *V. dahliae* in cotton. The overexpression of *GaGSTF9* significantly increased the resistance to Verticillium wilt in Arabidopsis; the *gstf9* mutant of Arabidopsis was more susceptible to Verticillium wilt than WT plants. These results suggest that GSTs may be a candidate family for investigating cellular mechanisms against *V. dahliae*. Overall, our study provides valuable information for breeding Verticillium wilt-resistant cotton varieties and for further research into the roles of GSTs.

Materials and Methods

Disease resistance identification and growth conditions

We obtained 215 accessions of *G. arboreum* L. from the Chinese National Mid-term Gene Bank and used these in a preliminary infection assay to identify those showing most resistance and most susceptibility to *V. dahliae* Kleb. wilt. The 215 accessions were planted in paper pots using a completely randomized block design. We classified the accessions using a five-scale DI for sensitivity to *V. dahliae* disease by exposing seedlings as previously described (Zhu et al. 2013). Three independent repeats of the infection assay were carried out with five seeds per pot and six pots per accession in each replication. The plants were grown in a greenhouse at a constant temperature of 25 °C, under a 1 h/8 h light/dark photoperiod and 80% relative humidity. The assay identified the GA0073, GA0099 and GA0105 accessions as resistant and the GA0176, GA0202 and GA0206 accessions as susceptible; these accessions were used for verification of the function of the candidate gene identified in this study.

An overexpression vector, designated 35S::*GaGSTF9*, was transformed into WT *A. thaliana* (L.) Heynh. Col-0. Seedlings of the T_4 generation of the single-copy insertion line OE4 had stable and high expression of *GaGSTF9* and were used in disease-related experiments. The Arabidopsis T-DNA line (Salk_001519C, *AtGSTF9*) was obtained from the Arabidopsis Biological Resource Center (ABRC). *Arabidopsis thaliana* was grown in a greenhouse at 22 °C, under a 16 h/8 h light/dark photoperiod and 60% relative humidity.

Culture and inoculation of *V. dahliae*

The antagonistic defoliating Vd07038 isolate of *V. dahliae* was cultured on potato dextrose agar medium at 25 °C for 6 d. Colonies were then inoculated into Czapek's medium (3% sucrose, 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.002% $FeSO_4 \cdot 7H_2O$ and 0.131% KH_2PO_4), and incubated at 25 °C for 5–7 d at 150 r.p.m. The conidial suspensions were then diluted with distilled water to a final concentration of about 1×10^7 ml^{-1}. A 10 ml aliquot of the conidial suspension was applied to the bottom of a plant pot to infect cotton seedlings.

Arabidopsis plants were grown for 20 d; 10 ml of conidial suspension was injected into the soil near their roots using a sterile needle. Control plants in every treatment were inoculated with an equal volume of sterile distilled water. Details of the in vitro inoculation technique, inoculation of the plate and assessment of the extent of stunting are provided by Veronese et al. (2003). Drops (2 µl) of conidial suspension (5×10^3 conidia ml^{-1}) were used to inoculate the roots of 2-week-old seedlings.

Genome-wide association study

Genotypic data with an average 6-fold sequencing depth were obtained from a previously unpublished study by our team; these sequencing data have been deposited in the NCBI Sequence Read Archive under accession No. SRP092170. DI frequency distributions were illustrated by GraphPad Prism 5.0 software using a frequency histogram (the center of the first bin equals 5 and bin width equals 10). We performed the GWAS for Verticillium wilt using 1,568,133 high-quality SNP markers with a minor allele frequency >0.05 and missing rate <40%. The Efficient Mixed-Model Association eXpedited (EMMAX) software (Kang et al. 2010) was used for GWAS. We used the kinship matrix in the emmax-kin-intel package of EMMAX to model population stratification and hidden relatedness. Then we used the formula $P = 1/N$ (where N is the effective number of independent SNPs) to evaluate the genome-wide significance thresholds in this study (Li et al. 2012). The significant −log (P) was about 5. Quantile–quantile (QQ) plots and Manhattan plots were visualized following the method described by Turner (2014).

Haplotype block analysis

We extracted the SNPs locating within 1 Mb distance on either side of SNP Ca3_23037225 from the vcf file and calculated the haplotype block using TASSEL (version 5.2.22) by default parameter (Bradbury et al. 2007).

Virus-induced gene silencingand construction of the overexpression vector

Total RNAs were extracted from the leaves of plants from the GA0099 accession using an RNAprep Pure Plant Kit (Tiangen) and used to generate cDNAs with a PrimeScript 1st Strand cDNA Synthesis Kit according to the manufacturer's instructions (TAKARA). For the VIGS experiments, the primer pair VGaGSTF9-F/VGaGSTF9-R, shown in **Supplementary Table S3**, was used to amplify the *GaGSTF9* fragment (398 bp). The PCR product was digested with *Xba*I and *Sac*I (BioLabs) and recovered using a Wizard SV Gel and PCR Clean-Up System (Promega). The recovered DNA was inserted into a TRV-based vector using TRV::*CLA1* (cloroplastos alterados 1 gene) as a positive control (Gao et al. 2011). To construct the overexpression vector, the primer pair GaGSTF9-F and GaGSTF9-R was used to amplify the full-length coding sequence (645 bp) of *GaGSTF9*, and *Xba*I and *Asc*I were used to insert the sequence into a T-vector (TAKARA) (**Supplementary Table S3**). *Xba*I and *Asc*I (BioLabs) were used to digest T-GaGSTF9 and pCAMBIA3300 (Cambia), and small and large fragments

were recovered. The recovered fragments were linked using T4 ligase following the manufacturer's instructions (TAKARA). All the vectors used in this study were transformed into *Agrobacterium tumefaciens* strain GV3101 using the freeze–thaw method (Yothishwaran et al. 2007).

Agrobacterium infiltration

The VIGS experiments were carried out using the methods described by Pang et al. (2013). Cotton seedlings with two fully expanded cotyledons were used for the *Agrobacterium* infiltration. Ten days after transformation, the cotton seedlings were inoculated with *V. dahliae*. Tissues were sampled at 20 and 25 d.p.i. The tissues for the relative expression analysis were rapidly frozen in liquid nitrogen and stored at −80 °C. Transcript levels were analyzed in three leaves from each of three independent plants of TRV::*GaGSTF9*. At least 30 strains were used to carry out the DI survey, and at least three biological replicates were performed for each experiment.

Arabidopsis transformation and molecular analysis

Agrobacterium strain GV3101 containing the *GaGSTF9* overexpression vector was used to transform the ecotype Col-0 of *A. thaliana* by the floral dip method (Clough and Bent 1998). T_0 transgenic seeds were obtained and spread evenly in a pot. After 1 week, seedlings were sprayed with Basta solution (0.1%) to select positive transformants, with false-positive seedlings turning yellow and then dying. Transgenic seeds of the T_1 generation were selected on plates of Murashige and Skoog (MS) medium containing Basta. After a few days of growth, lines with segregation ratios of approximately 3 : 1 [Basta resistant : Basta sensitive] were selected for continuation to the T_2 generation. Similarly, transgenic seeds of the T_2 generation were grown on MS medium containing Basta to select T_3 homozygous lines. T_3 lines containing the transgene and exhibiting the correct segregation ratio were confirmed using qRT-PCR analysis of *GaGSTF9* expression. To examine the genomic organization of *GaGSTF9*, we performed a Southern blot analysis using total genomic DNA extracted from young leaves of transgenic plants as previously described (Zhang et al. 2011). Only stable homozygous plants of the T_4 generation that showed high expression levels were chosen for further functional analysis experiments. At least 30 strains were used to carry out the disease index survey, and at least three biological replicates were performed for each experiment.

Quantitative real-time-PCR

We extracted total RNAs from cotton roots and leaves, and leaves of WT and transgenic Arabidopsis plants using an RNAprep Pure Plant Kit (Tiangen). We then used a PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time, TAKARA) for production of cDNAs. The *G. arboretum his3* gene (cotton_A_11188) was used as an internal control gene for cotton, while *ubiquitin 10* (*UBQ10*) (accession No. At4g05320) was used as the internal control for Arabidopsis. We used Primer Premier 6.0 software (Premier) to design all qRT-PCR primers (**Supplementary Table S3**). Diluted cDNA was used for qRT-PCR with SYBR Premix Ex Taq (Tli RNaseH Plus, TAKARA) on an ABI 7900 qRT-PCR System (Applied Biosystems). A three-step method was used with the following PCR conditions: 40 cycles at 95 °C for 30 s, 95 °C for 5 s and 60 °C for 30 s. We checked the dissociation curves of each reaction and used the cycle threshold (CT) $2^{-\Delta\Delta^{CT}}$ method (Livak and Schmittgen 2001) to calculate the expression level of each target gene. Each reaction was performed with at least three biological replicates.

Quantification of *V. dahliae* colonization

We used a previously described qRT-PCR approach to detect and quantify *V. dahliae* colonization. The primer pair V-QPCR-F and V-QPCR-R (**Supplementary Table S3**) and the qRT-PCR program were used as previously described (Atallah et al. 2007).

MeJA, SA and ET treatments

Solutions of 1 mM MeJA, SA and ET (ethephon solution rather than ethylene) were used for plant treatments. Cotton seedlings or Arabidopsis seedlings were cultured in pots in a greenhouse and sprayed with the different solutions at the foliage stage. Control (CK) plants were sprayed with water at the same pH.

Measurement of SA, GST and H_2O_2 levels

The levels of the immune system-related molecules SA, GST and H_2O_2 were monitored using various methods. Free SA content was determined using HPLC (Rigol L3000) as previously described (Dewdney et al. 2000). For GST and H_2O_2 quantitation, leaf samples were ground into powder in liquid nitrogen and analyzed with a Quantitative Assay Kit (Nanjing Jiancheng) according to the manufacturer's instructions. The concentration of GSH in the reaction system was reduced by 1 μmol l^{-1} as a unit of enzyme activity per milligram of protein at 37 °C for 1 min (less non-enzymatic reaction).

Verticillium dahliae recovery assay

To analyze the rates of infection of cotton and Arabidopsis by *V. dahliae*, we carried out an experiment using stem fragments from the first node of the stem. The length of stem from cotton was 4.5 cm and that from Arabidopsis was 3 cm. We used a previously described cleaning method (Zhang et al. 2011), sliced the stem sections into six or seven pieces and then plated them on potato dextrose agar plates. The plates were incubated at 25 °C and differences in *V. dahliae* infection were determined using the number of fungal growths on the stem sections of different lines.

Trypan blue staining

Plant cell death was visualized with trypan blue staining by a modified version of the previously described protocol (Choi and Hwang 2011). Leaves were soaked in trypan blue dye (1 g phenol, 1 mg trypan blue, 1 ml of lactic acid and 1 ml of glycerol dissolved in 1 ml of sterile distilled water) and then stained by boiling. After cooling to room temperature, samples were decolorized with a chloral hydrate solution (2.5 g ml^{-1}).

Disease index, stunting and assessment of chlorosis

The health status of plants is generally measured using a DI, which provides a comprehensive and objective assessment; a higher DI indicates more serious infection. For cotton, DI was calculated using the formula:

$$\text{DI}\ (\%) = [\Sigma\text{S disease grades} \times \text{number of infected plants})/(\text{total checked plants} \times 4)] \times 100.$$

Seedlings were classified into five grades (grade 0, 1, 2, 3 and 4) according to symptoms manifested on the cotyledons and true leaves (Wang 2004, Zhang et al. 2012). For Arabidopsis, the disease severity of plants was graded from 0 to 5, and the DI was calculated according to the following formula:

DI (%) = [(Σ disease grades × number of infected plants)/(total checked plants × 5)] × 100, as described previously (Veronese et al. 2003).

The extent of stunting was rated on a scale of 0–3 in which 0 = no stunting, 1 = moderate reduction of leaf area, 2 = strong reduction of leaf area and 3 = strong reduction of leaf area, leaf number and length of stem.

The extent of leaf chlorosis was rated on a scale of 0 to 4 in which 0 = no symptoms, 1 = <25% chlorotic leaves, 2 = 25–50% chlorotic leaves, 3 = 50–75% chlorotic leaves and 4 = 75–100% chlorotic leaves (Veronese et al. 2003).

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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