Transcriptome analysis reveals the cotton defense mechanism in response to *Verticillium dahliae* in the presence of the biocontrol fungus *Chaetomium globosum* CEF-082

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Abstract

Background: Verticillium wilt of cotton is a serious soil-borne disease that causes a substantial reduction in cotton yields. A previous study showed that the endophytic fungus Chaetomium globosum CEF-082 could control Verticillium wilt of cotton, and induce a defense response in cotton plants. However, the comprehensive molecular mechanism governing this response is not clear.

Results: To study the signalling mechanism induced by CEF-082, the transcriptome of cotton seedlings pretreated with CEF-082 was sequenced. The results revealed 5638 DEGs at 24 h post inoculation with CEF-082, and 2921 and 2153 DEGs at 12 and 48 h post inoculation with Verticillium dahliae, respectively. At 24 h post inoculation with CEF-082, KEGG enrichment analysis indicated that the DEGs were enriched mainly in the plant-pathogen interaction, MAPK signalling pathway-plant, flavonoid biosynthesis, and phenylpropanoid biosynthesis pathways. There were 1209 DEGs specifically induced only in cotton plants inoculated with V. dahliae in the presence of the biocontrol fungus CEF-082, and not when cotton plants were only inoculated with V. dahliae. GO enrichment analysis revealed that these DEGs were enriched mainly in the following terms: ROS metabolic process, H2O2 metabolic process, defense response, superoxide dismutase activity, and antioxidant activity. Moreover, many genes, such as ERF, CNGC, FLS2, MYB, GST and CML genes, were identified that regulate crucial points in defence-related pathways and that may contribute to V. dahliae resistance in cotton. These results provide a basis for the understanding of the molecular mechanism by which biocontrol fungus CEF-082 increased the resistance of cotton to Verticillium wilt.

Conclusions: The results of this study showed that CEF-082 could regulate multiple
metabolic pathways in cotton. After treatment with V. dahliae, the defense response of cotton plants preinoculated with CEF-082 was strengthened.

Background

Cotton (Gossypium spp.) is an important economic crop species cultivated worldwide. Verticillium wilt of cotton is a serious vascular disease that detrimentally affects cotton yield and fibre quality [1]. Verticillium wilt is caused by the soil-borne fungus Verticillium dahliae Kleb. This disease can cause cotton yellowing, wilting, defoliation, and ultimately death [2]. It is difficult to control this pathogen because of its long-term survival as microsclerotia in the soil and its broad host range [3]. To date, no fungicide has been identified that can eliminate Verticillium wilt of upland cotton (Gossypium hirsutum L.) after plants have been infected [2, 4-5]. At present, the use of biological control agents is a promising, more environmentally friendly strategy to control Verticillium wilt of cotton [6]. Numerous studies have shown that various biological control agents can suppress Verticillium wilt in different hosts species [7-8]. Iturins mediate the defense response, and significantly activate PR1, LOX, and PR10 at 24 h after V. dahliae infection[9]. The nonvolatile substances produced by CEF-818 (Penicillium simplicissimum), CEF-325 (Fusarium solani), CEF-714 (Leptosphaeria sp.), and CEF-642 (Talaromyces flavus) inhibit V. dahliae growth [10]. Fusarium oxysporum 47 (Fo47) reduced the symptoms of Verticillium wilt in pepper; the expression of three defense genes, CABPR1, CACHI2 and CASC1, was upregulated in the roots[11]. Bacillus subtilis DZSY21 reduced the disease severity of southern corn leaf blight, and upregulated the expression level of PDF1.2 [12]. Preinoculation of cauliflower with Verticillium Vt305 reduced symptom development and the colonization of plant tissues by
Verticillium longisporum [13]. Various fungal and bacterial strains showed biocontrol activity against Verticillium wilt of olive. These microorganisms protect plants from the deleterious effects of the various pathogens, cause induced systemic resistance (ISR), compete for nutrients and colonization space, or promote plant growth by the production of phytohormones and the delivery of nutrients [14].

It has been reported that a series of immune reactions are induced in cotton plants infected with V. dahliae. In recent years, transcriptomic studies of the defence responses of plants infected with V. dahliae have become increasingly common, and several signal transduction pathways and key genes have been identified, including those involved in plant hormone signal transduction, plant-pathogen interaction, and phenylpropanoid-related and ubiquitin-mediated signals in cotton; additionally, these studies have investigated members of key regulatory gene families, such as receptor-like protein kinases (RLKs), WRKY transcription factors and cytochrome P450s (CYPs) [3]. The expression levels of phenylalanine ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL), cinnamyl alcohol dehydrogenase (CAD), caffeoyl-CoA O-methyltransferase (CCoAOMT), and caffeoyl O-methyltransferase (COMT) in the phenylalanine metabolism pathway have been shown to be upregulated in sea-island cotton [2]; the expression levels of 401 transcription factors (TFs), mainly within the MYB, bHLH, AP2-EREBP, NAC, and WRKY families, have been shown to be up- or downregulated in response to V. dahliae in Arabidopsis thaliana [15]; and genes encoding cyclic nucleotide gated channel (CNGC), respiratory burst oxidase homologue (RBOH), flagellin-sensitive 2 (FLS2), jasmonate ZIM domain-containing protein (JAZ), transcription factor MYC2, regulatory protein NPR1 and transcription factor TGA have been shown to be induced by V. dahliae in sunflower[16]. Several studies have investigated transcript
levels in plants in response to biocontrol agents, but these studies have mainly involved *A. thaliana*. An analysis of gene expression changes in *A. thaliana* in the presence of *Trichoderma harzianum* T34 revealed that salicylic acid (SA) - and jasmonic acid (JA)-related genes were down-regulated [17]. *PDF1.1*, a putative defensin, was upregulated after *A. thaliana* was treated with *Bradyrhizobium* sp. strain ORS278 independently of pathogen attack [18].

In previous studies, we found that the endophytic fungus *Chaetomium globosum* CEF-082 isolated from upland cotton plants suppressed the growth of *V. dahliae* and increased cotton resistance to Verticillium wilt [19]. However, the signalling mechanism induced by CEF-082 is unknown. Therefore, the purpose of this study was to reveal the molecular mechanism by which CEF-082 increased cotton resistance to Verticillium wilt via RNA sequence analysis.

**Methods**

**Fungal strain culture**

Endophytic *C. globosum* CEF-082 of cotton was cultured on potato dextrose agar (PDA) plates for 20 d. Spores were obtained by adding sterile water to each plate, rubbing a sterile spatula over the colony and then filtering the suspension through a sterile cheesecloth, after which the suspension was diluted to a $1 \times 10^5$ spore/mL. *V. dahliae* VD1070-2 was cultured on PDA for 7 d, inoculated into liquid Czapek-Dox medium[20], and cultured in the dark at 25°C and 150 rpm for 7 d. The mycelia were filtered out and removed, and the filtrate was subsequently diluted to a $1 \times 10^7$ spore/mL spore suspension.

**Cotton inoculation treatment**

Jimian 11, a highly Verticillium wilt-susceptible upland cotton variety, was provided
by Professor Heqin Zhu from State Key Laboratory of Cotton Biology, Institute of Cotton Research of Chinese Academy of Agricultural Sciences. It is a hybrid [(Jihan 4× Ke 4104) F_2 × 74Yu102]. The seeds were sterilized with 70% alcohol for 1 minute and then with 1.05% sodium hypochlorite for 10 minutes, after which the seeds were washed with sterile water 5 times. The cotton seeds were planted in vermiculite and transferred to plastic pots (25 cm × 15 cm) that contained 2000 mL of liquid culture solution after emergence. The cultivation solution was prepared according to the methods of Zhang et al. [21], with some modifications. In this study, 2 mM NaCl was used instead of 2.5 mM KCl, while the other 9 mineral nutrients were the same. A black cystosepiment with 20 holes was placed on the plastic pot, and cotton plants were placed into the holes and supported by a sponge. Twenty plants were cultivated per pot per treatment, and each treatment was repeated three times. Twenty cotton plants in each treatment were removed from the plastic pots, and inoculated with CEF-082 by soaking the cotton roots in 300 mL of a 1×10^5 spore/mL spore suspension for 40 minutes prior to the first true leaf flattening. Instead of the CEF-082 spore suspension, water was used as the control group. The cotton plants were then returned to the pots. At 0 h, 6 h and 24 h later, 5 leaves were randomly taken at each time point for each biological replicate in each treatment, and 24 h was considered 0 h before inoculation with V. dahliae (24 h (0 h)). Twenty four hours post inoculation with CEF-082, the same method was used to inoculate V. dahliae VD1070-2 (1×10^7 spore/mL) in the treatment group and the control group. Leaf samples were then collected at 12 h, 1 d, 2 d, 3 d, 5 d and 7 d, and 5 leaves were also randomly collected at each time point for each biological replicate under each treatment. Three biological replicates
were included.

Determination of the hydrogen peroxide (H$_2$O$_2$) content

H$_2$O$_2$ content was estimated according to the methods of Sharma A et al. [22] with minor modifications. Approximately 0.1 g of cotton leaves was weighed and added to 1 mL of acetone for ice bath homogenization. The samples were then centrifuged at 8000 g and 4°C for 10 minutes, and the supernatant was collected. Then, 25 μL of 20% titanium chloride in concentrated HCl and 200 μL of ammonia solution (17 M) were added. The precipitate was then washed 3 times with acetone. Afterward, the washed precipitates were dissolved in 1.5 mL of H$_2$SO$_4$ (2 N), and the absorbance was read at 415 nm.

Control effect of the biocontrol fungus CEF-082 on Verticillium wilt of cotton

The above hydroponic seedlings were investigated at 14 d post inoculation (dpi) with VD1070-2. The disease severity was rated according to a disease index that was based on a five-scale categorization of Verticillium wilt disease of cotton seedlings [23].

RNA sequencing (RNA-seq)

A polysaccharide polyphenol RNA extraction kit (TianGen, Beijing) was used to extract RNA from cotton leaves. Electrophoresis was performed, and a One Drop (1000+) spectrophotometer was used to detect the concentration and quality of RNA. Transcriptome sequencing was performed for the 24 h (0 h (T0h, C0h)), 12 h (T12h, C12h) and 48 h (T48h, C48h) samples. T0h, T12h and T48h represented 0, 12 and 48 h samples in the treatment group, respectively, and C0h, C12h and C48h represented 0, 12 and 48 h samples in the control group, respectively. Three biological replicates were performed, and there were 18 samples. The construction
of the DNA library and sequencing were performed by Beijing Genomics Institute (BGI). Data filtering was performed using SOAPnuke software (BGI, Beijing). Clean reads were obtained by removing the reads containing adapters, reads with more than 5% N, and low-quality sequences. The clean reads were spliced and aligned to the reference *G. hirsutum* genome retrieved from the cotton genome website (https://www.cottongen.org/). The FPKM (fragments per kilobase per transcript per million mapped reads) values were calculated and used to estimate the effects of sequencing depth and gene length on the mapped read counts.

Screening and analysis of differentially expressed genes (DEGs)

The DEGSeq R package (1.20.0) [24] was used to analyse DEGs in cotton leaves treated or nontreated with CEF-082 under the criteria of a corrected *P* value < 0.001 and an absolute log2 ratio ≥1. GO (Gene Ontology) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were enriched by DEGs if the *P* values were < 0.001. Resistance genes among the DEGs were predicted by a BLAST search of the Plant Resistance Gene (PRG) Database (identity ≥40, *E*-value < 1E-5) [25]. TFs coded by the DEGs were predicted (*E*-value < 1E-5) according to the Plant Transcription Factor Database [26].

Quantitative reverse-transcription-PCR (qRT-PCR) analysis

The plant-pathogen interaction pathway and R genes are important for plant resistance. Twelve DEGs involved in the plant-pathogen interaction pathway and predicted R genes were randomly selected for qRT-PCR to verify whether the trend of gene expression was consistent with the transcriptome sequencing results. Data were collected from three replicate experiments, and the samples used for qRT-PCR were the same as those used for RNA-seq. RNA was extracted from sample leaves and reverse transcribed into cDNA. qRT-PCR was performed via a CFX96 Real-Time
System (BioRad), and each PCR mixture (20 μL) consisted of 10 μL SuperReal PreMix Plus SYBR Green (Tiangen, China), 0.4 μL of each primer, 2 μL of cDNA and 7.2 μL of sterile water. Each sample involved at least three technical repeats. The PCR cycle consisted of an initial denaturation step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The cotton ubiquitin gene was used as the internal reference, and relative gene expression was calculated using the 2^{-ΔΔCT} method. Primers were obtained from the upland cotton gene fluorescence quantitative specific primer database (https://biodb.swu.edu.cn/qprimerdb/) (Table S1).

Results

Control effect of CEF-082 on Verticillium wilt of cotton and the H$_2$O$_2$ content

The disease index was 18.61 in the control group (water+ V. dahliae) and 7.62 in the treatment group (CEF-082+ V. dahliae) 14 d after V. dahliae inoculation (Fig. 1A). The results showed that CEF-082 enhanced the resistance of cotton to Verticillium wilt, and the biocontrol effect was 59.1% (Fig. 1C). The H$_2$O$_2$ content in the treatment group was higher than that in the control group throughout the majority of the duration of the experiment and lower than that in the control group at 5 dpi with V. dahliae. The H$_2$O$_2$ content in the treatment group was highest at 2 dpi (12.80 μmol/g), while the H$_2$O$_2$ content in the control group was highest at 1 dpi (10.38 μmol/g). The changes in the two groups were similar and were stable 5 d later (Fig. 1B).

Verification of RNA-Seq Analysis by qRT-PCR

Twelve DEGs were randomly selected. The gene expression levels in the control and
treatment groups were compared by qRT-PCR. The RNA-seq data showed that the expression of the 12 genes was upregulated at 0 h, 12 h or 48 h. The qRT-PCR results showed that the expression of nine of the 12 genes was upregulated, which was consistent with the results of their upregulated expression in the transcriptome; however, the expression of three genes was downregulated, which was inconsistent with the expression of genes identified in the transcriptome, namely, Gh_D12G2793, Gh_D08G2484 and Gh_D05G3615 (Fig. 2). In addition, the level of upregulation of 5 genes in the qRT-PCR data was lower than that in the RNA-seq data. The qRT-PCR data were up to 75% consistent with the transcriptome data.

Functional annotation and enrichment analysis of the DEGs

The minimum correlation between the three replicates was 95.5% (Fig. S1). Principal component analysis (PCA) of 18 arrays (Fig. S2) was also used to compare samples and to explore the dynamic changes in the cotton transcriptome after treatment with CEF-082 and *V. dahliae*.

The average clean reads of the 18 samples was 62.08 M. The lowest Q20 value of the clean reads was 97.93, and the lowest Q30 value was 90.06 (Table S2). A total of 47183 new transcripts were found, of which 7288 belonged to new protein-coding genes (Table S3).

There were 3480 upregulated and 2158 downregulated DEGs at 0 h, 1716 upregulated and 1205 downregulated DEGs at 12 h, and 1524 upregulated and 629 downregulated DEGs at 48 h. The greatest number of DEGs were identified after inoculation with CEF-082 for 24 h. After inoculation with *V. dahliae*, the number of DEGs gradually decreased.

DEGs induced only by CEF-082 at 0 h (*V. dahliae* had not been inoculated) After inoculation with CEF-082 for 24 h (0 h), 5638 DEGs were identified, and KEGG
pathway enrichment analysis revealed 15 significantly enriched pathways, including plant-pathogen interaction, MAPK signalling pathway-plant, flavonoid biosynthesis, phenylpropanoid biosynthesis, galactose metabolism, arachidonic acid metabolism, carotenoid biosynthesis, glutathione metabolism, sesquiterpenoid and triterpenoid biosynthesis, linoleic acid metabolism, other glycan degradation, glycosphingolipid biosynthesis - ganglio series, brassinosteroid biosynthesis, diterpenoid biosynthesis and sphingolipid metabolism (Q-value <0.05) (Table 1). In the plant-pathogen interaction pathway, there were 106 $FLS2$ genes, 88 upregulated and 18 downregulated; 7 $Rboh$ genes, 5 upregulated and 2 downregulated; 5 upregulated calcium-dependent protein kinase (CDPK) genes; 5 CNGC genes, 3 upregulated and 2 downregulated; and 57 glutathione S-transferase (GST) genes in the glutathione metabolism pathway, 49 upregulated and 8 downregulated (Fig. 3). These genes were related to the metabolism of reactive oxygen species (ROS) and $Ca^{2+}$. In the MAPK signalling pathway-plant pathway, 304 DEGs regulated 30 crucial points related to ROS, $Ca^{2+}$, abscisic acid (ABA), ethylene (ET), JA, $H_2O_2$ and FLS2. In the flavonoid biosynthesis pathway, the genes encoding chalcone synthase (CHS) and ferulate-5-hydroxylase (F5H) were induced. In the phenylpropanoid biosynthesis pathway, the key genes $PAL$ and $4CL$ were also induced. The GO enrichment analysis revealed that the 5638 genes were mainly enriched in 86 terms, including the intrinsic component of membrane, integral component of membrane, membrane part, membrane, catalytic activity, response to biotic stimulus, cell wall, oxidoreductase activity, defense response, response to stimulus, response to stress, and response to fungus (Q-value <0.001), and the first 15 terms are listed in Table 2. Of the 16 genes in the response to fungus term, 15 were
upregulated and 1 was downregulated. The GO classification showed that there were 18, 14 and 12 terms in biological process, cellular component and molecular function, respectively, and the KEGG classification indicated that the DEGs mainly belonged to the metabolism pathway (2856 DEGs).

DEGs co-induced by CEF-082 and *V. dahliae*

There were 463 shared DEGs at 12 h and 48 h that were significantly enriched in 6 KEGG pathways (Table 3). In the plant-pathogen interaction pathway, 29 DEGs regulated 8 crucial points, including CNGCs, calmodulin (CaM), FLS2, disease resistance protein RPS2 (RPS2), heat shock protein 90 kDa (HSP90), pto-interacting protein 1 (Pti1), disease resistance protein RPM1 (RPM1), and EIX receptor 1/2 (EIX1/2). In the phenylpropanoid biosynthesis pathway, 23 DEGs regulated 9 crucial points. In the flavonoid biosynthesis pathway, 12 DEGs regulated 8 crucial points. The enriched GO terms included terpenoid metabolic process, oxidoreductase activity, defence response, H$_2$O$_2$ metabolic process and ROS metabolic process terms.

DEGs induced only in cotton inoculated with *V. dahliae* in the presence of CEF-082

A total of 1209 specific DEGs were identified at 12 h and 48 h, which were induced only in cotton plants inoculated with *V. dahliae* in the presence of CEF-082, but not when cotton plants only inoculated with *V. dahliae*. The cluster thermogram showed the expression patterns of these genes at different stages (Fig. S3). KEGG classification showed that these DEGs mainly belonged to metabolism (672 DEGs) and were significantly enriched in 5 KEGG pathways, including flavonoid biosynthesis, indole alkaloid biosynthesis, MAPK signalling pathway-plant, plant-pathogen interaction, and phenylpropanoid biosynthesis (Table 4). GO classification showed that there were 14, 12 and 9 terms in the biological process, cellular
component and molecular function, respectively. GO enrichment indicated that these DEGs were enriched in ROS metabolic process (14 DEGs), $\text{H}_2\text{O}_2$ metabolic process (12 DEGs), $\text{H}_2\text{O}_2$ catabolic process (12 DEGs), defense response (31 DEGs), superoxide dismutase activity (5 DEGs), antioxidant activity (19 DEGs), oxidoreductase activity, acting on superoxide radicals as acceptor (5 DEGs), cofactor binding (75 DEGs) and DNA binding (121 DEGs) (Fig. S4).

At 12 h and 48 h, 96 shared DEGs were obtained, which were induced only in cotton plants inoculated with $V.$ *dahliae* in the presence of CEF-082, but not when cotton plants only inoculated with $V.$ *dahliae* (Fig. S5). KEGG analysis of the 96 DEGs indicated that they were mainly enriched in glutathione metabolism and flavonoid biosynthesis (Table 5). GO analysis showed that the DEGs were enriched in superoxide dismutase activity, oxidoreductase activity, acting on superoxide radicals as acceptors, and antioxidant activity terms. Of the 96 DEGs, 9 encoded TFs and 20 encoded predicted PRGs (Table S4).

A protein-protein interaction network (Fig. S6) was constructed via the 96 DEGs shared between 12 h and 48 h and genes interacting with them in cotton. Six hub genes were obtained, including Gh_A05G1020, Gh_D09G0858, BGI_novel_G004376, Gh_A08G0125, Gh_D07G1197 and Gh_A05G3508. Among them, Gh_D07G1197 was enriched in the flavonoid biosynthesis pathway.

Putative R genes and TFs involved in resistance to *Verticillium* wilt

On the basis of the transcriptome analysis, a total of 65 candidate genes that may be related to the resistance of cotton to *Verticillium* wilt were identified, including 5 CNLs (whose members contain an NB-ARC domain), 3 CNs (members of the U-box domain-containing protein kinase family protein), 5 NLs (whose members contain an NBS-LRR domain), 7 RLPs (whose members contain an eLRR-TM-S/TPK domain), 7 Ns
(whose members contain an NBS domain only), 9 TNLs (members of the TIR-NBS-LRR class), 6 Ts (members of NAC domain containing protein 17), 1 Mlo-like (a member of the Mlo-like resistant proteins) and 2 other types (which have resistance functions but do not fit the known classes). These genes mainly included a disease resistance protein, 2 probable calcium-binding protein (CML45), 3 ethylene-responsive transcription factor (ERF), 2 cyclic nucleotide-gated ion channel 2 (CNGC2), 5 MYB TFs and 2 GST (Table 6-1, Table 6-2, and Table 6-3). Clustering thermogram of 65 genes (Fig. 4) showed that certain genes were upregulated at 0, 12 and 48 h; certain genes were downregulated at 0 h, while upregulated at 12 and 48 h; certain genes were downregulated at 0, 12 and 48 h.

Discussion

The number of DEGs identified at 12 h and 48 h was lower than that identified at 0 h. The number of DEGs may have decreased because both plants were infected with *V. dahliae* and began to respond defensively. For CEF-082 treatment and CEF-082+ *V. dahliae* treatment, DEGs were enriched mainly in 5 signalling pathways, plant-pathogen interaction, MAPK signalling pathway-plant, flavonoid biosynthesis, phenylpropanoid biosynthesis, and glutathione metabolism. The pathways of plant-pathogen interaction and flavonoid biosynthesis were also induced in sunflower infected with *V. dahliae*[16], and the results were also consistent with those of Tan [27], who reported that most DEGs in tomato were associated with phenylpropanoid metabolism and plant-pathogen interaction pathways. However, the glutathione metabolism pathway has rarely been reported in the transcriptome of cotton plants treated with *V. dahliae*.

It is clear that plant responses to biotic or abiotic stress depend on interactions
among several signalling pathways, including those mediated by JA, ET, SA or ABA [28-29]. Morán-Diez E et al. [17] found SA- and JA-related DEGs were down-regulated in A. thaliana after 24 h of incubation in the presence of T. harzianum T34. A set of DEGs influenced by JA or ET, was induced upon pathogen attack when A. thaliana were previously colonized by a photosynthetic Bradyrhizobium sp. strain, ORS278 [18]. DEGs related to ET, SA, JA, brassinosteroid (BR) and cytokinin were upregulated or downregulated upon V. dahliae infection in cotton [3]. In this study, we also found that DEGs in ABA, auxin and gibberellin were significantly induced not only after treatment with CEF-082 but also after inoculation with V. dahliae.

Besides, DEGs related to JA, ET, SA, BR and cytokinin were induced in cotton plants treated only with CEF-082. The 8 plant hormones were also induced after infection with V. dahliae in sunflower [16]. The responses of the A. thaliana auxin receptors TIR1, AFB1 and AFB3 and auxin transporter AXR4 were impaired upon infection with V. dahliae [30]. Therefore, both CEF-082 and V. dahliae can induce changes in hormones.

Previously, it was shown that after plants were infected with pathogens, the FLS2 pattern recognition receptors recognized pathogens, and the hypersensitive response (HR) was activated through ROS, JA, WRKYs and the NO signalling pathways [31-32]and mediated by CNGC, RBOH, CaM/CML and FLS2[33-35]. These results are consistent with the results from this study. In this study, 24 h after treatment with CEF-082, the DEGs of FLS2, Rboh, CDPK, CNGCs and GST in the plants were also upregulated or downregulated to varying degrees (Fig. 3). In addition, most of the genes coding peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) were also upregulated. These genes were related to the accumulation of ROS. Forty-eight hours after treatment with V. dahliae, the genes
encoding CNGC, CaM/CML and FLS2 were upregulated. However, in this study, the NO signalling pathway was not induced.

Phenylpropane synthesis is related to cotton defense mechanisms [36], while flavonoids are known to buffer substantial stress-induced alterations in ROS homeostasis and to modulate the ROS-signalling cascade[37]. Plant CNGC subunits and CaM constitute a molecular switch that either opens or closes calcium channels [38]. Previous reports have shown that calcium-dependent CDPK4 and CDPK5 regulate ROS production by phosphorylating NADPH oxidase in potatoes [39]. ROS are important not only for signalling mechanisms for defense [40] but also for regulating programmed cell death via the establishment of the HR [41]. MAPK family members can improve resistance to Verticillium wilt of cotton [42]. In this study, 24 h after CEF-082 inoculation, certain signal transduction pathways might have been involved in the plant response to CEF-082 (Fig. 5). After inoculation with CEF-082, FLS2 recognized CEF-082, MAPK signal transduction was induced, and calcium channels opened. \( \text{H}_2\text{O}_2 \) was then produced, leading to an ROS burst. Plant hormones were also induced, including ET, SA, JA, ABA, BR, auxin, gibberellin and cytokinin. The signalling pathways of flavonoids and phenylpropane synthesis were also involved in this process. In addition, lignin synthesis was also induced after treatment with CEF-082 (Fig. 6). Fig. 6 referred to the lignin biosynthesis pathway of Miedes et al.[43]. \textit{Cinnamate 4-hydroxylase (C4H)} and \textit{p-coumarate 3 hydroxylase (C3H)} were not induced in T0h-vs-C0h, T12h-vs-C12h, or T48h-vs-C48h but were induced in C12h-vs-C0h, which was similar to the results of Xu et al. [44], who indicated that \textit{C4H-1} and \textit{C4H-3} were upregulated after treatment with \textit{V. dahliae}. Three days after inoculation with \textit{V. dahliae}, lignin was detected, and the pith diameter of CEF-082 + \textit{V. dahliae}-treated plants was slightly larger than that of
water + *V. dahliae*-treated plants (Fig. S7). The defense response at T12h and T48h was similar to that at T0h, and only some key points induced were different in the pathways, which are shown in Fig. 11 and Fig. 12. Thus, it is speculated that CEF-082 reduced the occurrence of cotton Verticillium wilt because inoculation with CEF-082 can prime signalling pathways in defense against *V. dahliae* upon infection.

When pathogens infect plants, they induce a series of defense responses. GST participates in plant defences and can remove ROS[45]. Plant GSTs can be subdivided into eight categories, phi, zeta, tau, theta, lambda, dehydroascorbate reductase (DHAR), elongation factor 1 gamma (EF1G) and tetrachlorohydroquinone dehalogenase (TCHQD) [46]. *GSTF8* was used as a marker in early stress and defense responses [47], and JA, methyl jasmonate, ABA and H$_2$O$_2$ can induce GST expression [48-50]. *LrGSTU5* was obviously upregulated after treatment with *Fusarium oxysporum* [51], and the GST genes were also upregulated in *G. barbadense* treated with *V. dahliae* [52]. In this study, the GST genes were also significantly induced 24 h after treatment with CEF-082 (Fig. 5), and GST genes were upregulated in cotton treated with Water + *V. dahliae*. These results are consistent with those of Han et al. and Zhang et al. [51-52]. Certain GST genes were also significantly induced in the treatment group but were not significantly induced in the control group after treatment with *V. dahliae*. The GST gene *Gh_A09G1509* was shown to increase resistance to Verticillium wilt in tobacco[53]. Hence, we suggest that CEF-082 can induce specific GST genes to protect cotton from *V. dahliae*.

*V. dahliae* can induce a defense response after it infects cotton [3]. In this study, susceptible cotton varieties were inoculated with the biocontrol fungus CEF-082 and *V. dahliae*, which also induced a series of defence responses. Compared with plants
inoculated with water +V. dahliae, the plants inoculated with CEF-082 + V. dahliae presented significantly upregulated or downregulated expression levels of resistance-related genes. Therefore, it is speculated that the defense response was strengthened after inoculation with the biocontrol fungus CEF-082. In addition, we obtained 1209 specific DEGs, which could not be induced in plants inoculated with water +V. dahliae, and induced only in plants inoculated with CEF-082 +V. dahliae. GO enrichment showed that these genes were involved in the metabolic process of ROS. The disease resistance of cotton was enhanced after CEF-082 treatment, and thus, we inferred that these specific DEGs might be genes related to plant disease resistance.

Conclusions
CEF-082 can induce defense responses in cotton, and pretreatment with CEF-082 at an appropriate concentration of 10^5 spore/mL can improve the resistance of cotton (Jimian 11) to Verticillium wilt. Transcriptome analysis revealed that genes in cotton leaves involved in ROS burst, Ca^{2+}, lignin biosynthesis, flavonoids and phenylpropane synthesis were significantly upregulated or downregulated. Defense response could been induced in cotton plants treated with CEF-082, and in cotton plants inoculated with V. dahliae in the presence of CEF-082 was strengthened. Besides, 1209 specific DEGs were obtained, which induced only in plants inoculated V. dahliae in the presence of the biocontrol fungus CEF-082.

Abbreviations
FLS2: flagellin-sensitive 2; Rboh: respiratory burst oxidase homologue; CDPK: calcium-dependent protein kinase. CYP: Cytochrome P450 proteins; PAL:
phenylalanine ammonia-lyase; 4CL: 4-coumarate-CoA ligase; CAD: cinnamyl alcohol dehydrogenase; CCoAOMT: caffeoyl-CoA O-methyltransferase; COMT: caffeoyl O-methyltransferase; SA: salicylic acid; JA: jasmonic acid; ABA: abscisic acid; ET: ethylene; BR: brassinosteroid; POD: peroxidase; SOD: superoxide dismutase; CAT: catalase; ROS: reactive oxygen species; GST: glutathione S-transferase; CDPK: calcium-dependent protein kinase; RPS2: disease resistance protein RPS2; HSP90: heat shock protein 90kDa; Pti1: pto-interacting protein 1; CML: calcium-binding protein; HR: hypersensitive response; C4H: cinnamate 4-hydroxylase; C3H: p-coumarate 3 hydroxylase; ERF: ethylene-responsive transcription factor; CHS: chalcone synthase; F5H: ferulate-5-hydroxylase; qRT-PCR: Quantitative reverse-transcription-PCR.

**Declarations**

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**Authors’ contributions**

YZ, LZ, HZ and CT conceived the study. YZ and NY performed the experiments. YZ analysed the results and wrote the manuscript, with feedback from all authors. All authors have read and approved the manuscript.
Availability of data and materials

Most data supporting the results and conclusions are included in the article and additional files.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures
Figure 1

Disease index and symptoms of Verticillium wilt in cotton 14 d after V. dahliae inoculation.

(A) The disease index of cotton. (B) Content of H$_2$O$_2$ (pmol/g) over time. (C) Symptoms of Verticillium wilt in cotton: a: water + V. dahliae, b: CEF-082 + V. dahliae. Bars represent SEs.
Comparison of the expression trends of the qRT-PCR and RNA-seq data. The grey
Figure 3

Expression levels of genes related to ROS and Ca2+. The red color represents upregulation, and the green represents downregulation.
Figure 4

Clustering thermogram of putative R genes and genes encoding TFs.
Figure 5
Signal transduction pathways induced by CEF-082.

Figure 6
Lignin biosynthesis pathway [43]. Enzymes coloured in red or black indicate the k
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