Transcriptome analysis reveals the mechanism by which the biocontrol fungus Chaetomium globosum CEF-082 controls Verticillium wilt in cotton

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Research article

Keywords: Transcriptome, Verticillium wilt, Mechanism, Biocontrol fungi

Posted Date: October 11th, 2019

DOI: https://doi.org/10.21203/rs.2.15513/v1

Abstract

Background: Verticillium wilt of cotton is a serious soil-borne disease that causes a substantial reduction in cotton yield. A previous study showed that the endophytic fungus Chaetomium globosum CEF-082 could control Verticillium wilt of cotton, but the molecular mechanism by which CEF-082 controls Verticillium wilt is still unknown.

Results: To study the mechanism by which CEF-082 controls Verticillium wilt, the transcriptome of cotton seedlings pretreated with CEF-082 was sequenced. The results revealed 5638 DEGs 24 h post-inoculation with CEF-082, and 2921 and 2153 DEGs 12 and 48 h post-inoculation with Verticillium dahliae , respectively. At twenty-four hours post-inoculation with CEF-082, KEGG enrichment analysis indicated that the DEGs were mainly enriched in plant-pathogen interaction, MAPK signalling pathway-plant, flavonoid biosynthesis, and phenylpropanoid biosynthesis. There were 1209 DEGs specifically induced after inoculation with CEF-082 and V. dahliae . GO enrichment indicated that these DEGs were mainly enriched in the terms reactive oxygen species metabolic process, hydrogen peroxide metabolic process, defence response, superoxide dismutase activity, and antioxidant activity. Here, many genes, such as ERF, CNGC, FLS2, MYB, GST and CML, 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 fungi control Verticillium wilt.

Conclusions: In this study, we found that CEF-082 could regulate multiple metabolic pathways in cotton. After treatment with Verticillium dahliae , the defence response of cotton plants pre-inoculated with CEF-082 was strengthened.

Background

Cotton (*Gossypium* spp.) is an important economic crop that is 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, wilt, defoliation, and ultimately death [2]. It is difficult to control this pathogen due to its long-term survival as microsclerotia in the soil and its broad host range [3]. To date, no fungicide has been identified that can cure Verticillium wilt of upland cotton (*Gossypium hirsutum* L.) after plants have been infected [2, 4–5].

Many biocontrol bacteria and fungi have been studied to assess their ability to control Verticillium wilt and other plant diseases, and substantial progress has been made. Iturins mediate the defence response, and significantly activate *PR1, LOX,* and *PR10* at 24 h after *V. dahliae* infection [6]. 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 [7]. The application of the nonpathogenic isolate *Fusarium oxysporum* 47 (Fo47) reduced the symptoms of Verticillium wilt in pepper, and three defence genes, *CABPR1, CACHI2* and *CASC1,* were upregulated in the roots treated with

Fo47 [8]. K–165 induced the resistance of *Arabidopsis thaliana* to Verticillium wilt [9]. *Bacillus subtilis* DZSY21 reduced the disease severity of southern corn leaf blight and upregulated the expression levelof *PDF1.2* in DZSY21-treated plants [10]. The preinoculation of cauliflower with *Verticillium* Vt305 reduced symptom development and the colonization of plant tissues by *V. longisporum [*11]. However, the mechanism of the biological control of plant diseases remains unclear.

It has been reported that cotton plants infected with *V. dahliae* induce a series of immune reactions. In recent years, transcriptomic studies of the defence responses of plants infected with *V. dahliae* have becoming increasing common, and several signal transduction pathways and key genes have been identified, including plant hormone signal transduction, plant-pathogen interaction, and phenylpropanoid-related and ubiquitin-mediated signals in cotton; additionally, these studies have investigated the key regulatory gene families, such as receptor-like protein kinases (RLKs), WRKY transcription factors and cytochrome P450s (CYPs) [3]. *PAL, 4CL, CAD, CCoAOMT,* and *COMT* in the phenylalanine metabolism pathway have been shown to be upregulated in sea-island cotton [2]; 401 transcription factors, mainly in the MYB, bHLH, AP2-EREBP, NAC, and WRKY families, have been shown to be up- or downregulated by *V. dahliae* in *A. thaliana [*12]; and CNGC, RBOH, FLS2, JAZ, MYC2, NPR1 and TGA have been shown to be induced by *V. dahliae* in sunflower [13]. However, there are few studies on the transcriptome level in plants induced by biocontrol fungi or induced by biocontrol fungi and *V. dahliae* at the same time.

In previous studies, we found that the endophytic fungus CEF–082 isolated from upland cotton plants could control Verticillium wilt in cotton. However, the molecular mechanism of biocontrol is unknown. Therefore, the purpose of this study is to reveal the molecular mechanism by which CEF–082 controls Verticillium wilt in cotton via RNA sequence analysis to provide a basis for the understanding of the biological control of plant diseases.

Methods

Fungal strain culture

Endophytic *Chaetomium globosum* CEF–082 of cotton was cultured on potato dextrose agar (PDA) plates for 20 d. Spores were washed with sterile water and diluted to a 1×10^5 CFU/mL spore suspension. *V. dahliae* VD1070–2 was cultured on PDA for 7 d, inoculated into liquid Czapek-Dox medium [14], and cultured in the dark at 25°C and 150 rpm for 7 d. The mycelia were filtered out and diluted to a 1×10^7 CFU/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 with 1.05% sodium hypochlorite for 10 minutes and then washed with

sterile water 5 times. The cotton seeds were planted in vermiculite and transferred to a plastic pot (25 cm×15 cm) containing 2000 mL of culture solution after emergence. The culture solution was prepared according to the methods of Zhang et al. [15], 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. Twenty plants were cultured per pot. The cotton plants were inoculated with the CEF–082 spore suspension by soaking the cotton roots in the spore suspension for 40 minutes prior to the first true leaf flattening, and water, instead of the CEF–082 spore suspension, was used as the control group. Zero, 6 and 24 h later, leaf samples were taken, and 24 h was considered 0 h before inoculated with *V. dahliae.* After that, the same method was used to inoculate *V. dahliae* VD1070–2 into the treatment group and the control group. Then, leaf samples were collected at 12 h, 1 d, 2 d, 3 d, 5 d and 7 d.

Determination of the hydrogen peroxide (H_2O_2) content

H_2O_2 content was estimated according to Anket Sharma et al. [16] with minor modifications. Approximately 0.1 g of cotton leaves was weighed and added to 1 mL of acetone for ice bath homogenization. Then, the samples were 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. The washed precipitates were dissolved in 1.5 mL of H_2SO_4 (2 N), and the absorbance was read at 415 nm.

Control effect of 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 method of investigation was consistent with that of Zhu et al. [17].

RNA-seq

A polysaccharide polyphenol RNA extraction kit (TianGen) was used to extract RNA from cotton leaves. Electrophoresis was performed, and Drop one 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. Three replicates were performed, and there were 18 samples. The construction of the DNA library and sequencing were performed by BGI company. The raw reads obtained from sequencing were filtered to obtain clean reads, which were spliced and compared to the reference genome.

Screening and analysis of differentially expressed genes (DEGs)

To improve the accuracy of the identification of DEGs, we defined DEGs with a fold change > = 2 and Q-value < 0.001 as significant. Kyoto Encylopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses of these genes were carried out.

Principal component analysis (PCA)

The princomp function in R software was used for PCA analysis, and the ggplot2 package in R software was used to draw figures.

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

Some genes were selected for RT-PCR to calculate whether the trend of gene expression was consistent with the transcriptome sequencing results. RNA was extracted from sample leaves and re-transcribed into cDNA. Primers were obtained from the upland cotton gene fluorescence quantitative specific primer database (https://biodb.swu.edu.cn/qprimerdb/) (Table S1), and gene expression was calculated by $2^{-\Delta Ct}$.

Results

Control effect of CEF–082 on Verticillium wilt of cotton and the H_2O_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 could control Verticillium wilt of cotton, and the control effect was 59.1% (Fig. 1C).

The H_2O_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_2O_2 content in the treatment group was highest at 2 dpi (12.80 μmol/g), while the H_2O_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).

PCA

The minimum correlation between the three replicates was 95.5% (Fig. S1). According to the results of the PCA, at 0 h and 12 h, the gene expression difference between the control group and the treatment group was relatively small, but there was a big difference at 48 h (Fig. 2). These results revealed a gene expression difference between the CEF–082-treated group and the non-CEF–082-treated group.

qRT-PCR

RNA was extracted from cotton transcriptome samples and reverse transcribed into cDNA. Twelve DEGs were selected. The gene expression levels in the control and treatment groups were compared by qRT-PCR. The results showed that nine of the 12 genes were upregulated, which was consistent with the results of their upregulated expression in the transcriptome, while three genes were downregulated, which was inconsistent with the expression of the transcriptome genes, namely, Gh_D12G2793, Gh_D08G2484 and Gh_D05G3615 (Fig. 3). 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 consistent with the transcriptome data up to 75%.

Functional annotation and enrichment analysis of the DEGs

The average clean reads of the 18 samples was 62.08. 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 (Fig. 4). The highest number of DEGs were identified after inoculation with CEF–082 for 24 h. After inoculation with *V. dahliae,* the number of DEGs gradually decreased.

Effect of CEF–082 treatment on cotton seedlings

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 CDPK genes; 5 CNGC genes, 3 upregulated and 2 downregulated; and 57 GST genes in the glutathione metabolism pathway, 49 upregulated and 8 downregulated (Fig. 5). 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), jasmonic acid (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, defence 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 (Fig. 6) 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, RPS2, HSP90, Pti1, RPM1, and 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, hydrogen peroxide metabolic process and reactive oxygen species metabolic process terms.

DEGs specifically induced by CEF–082

A total of 1209 specific DEGs were identified at 12 h and 48 h after removing all of the shared DEGs in the three stages in the control group, and the cluster thermogram showed the expression patterns of these genes at different stages (Fig. 7). 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 reactive oxygen species metabolic process (14 DEGs), hydrogen peroxide metabolic process (12 DEGs), hydrogen peroxide catabolic process (12 DEGs), defence 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. S2).

At 12 h and 48 h, 96 shared DEGs were obtained by eliminating the shared DEGs in the CK at different stages (Fig. 8). 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, there were 9 transcription factors (TFs) and 20 plant resistance genes (PRGs) (Table S4).

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

Based on the transcriptome analysis, a total of 65 candidate genes were identified that may be related to the resistance of cotton to Verticillium wilt, including 5 CNLs, 3 CNs, 5 NLs, 7 RLPs, 7 Ns, 9 TNLs, 6 Ts, 1 Mlo-like and 2 other types. 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 glutathione S-transferase (GST) (Table 6–1, Table 6–2, and Table 6–3). Clustering thermogram of 65 genes was made (Fig. 9), and the results 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.

Protein interaction network induced by CEF–082

A protein-protein interaction network (Fig. 10) was obtained by using the 96 DEGs shared at 12 and 48 h and genes interacting with them in cotton. Six hub genes were obtained: 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.

Discussion

The number of DEGs identified at 12 h and 48 h was lower than that identified at 0 h. It has been suggested that the number of DEGs 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 mainly enriched 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 [*13], and the results were also consistent with those of Tan [18], 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.*

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.* The 8 plant hormones were also induced after infection with *V. dahliae* in sunflower [13]. The responses of the *A. thaliana* auxin receptors TIR1, AFB1 and AFB3 and auxin transporter AXR4 were impaired upon infection with *V. dahliae [*19]. 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 [20–21] and mediated by CNGC, RBOH, CaM/CML and FLS2 [22–24]. 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. 5). 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 defence mechanisms [25], while flavonoids are known were to buffer substantial stress-induced alterations in ROS homeostasis and to modulate the ROS-signalling cascade [26]. Plant CNGC subunits and CaM constitute a molecular switch that either opens or closes calcium channels [27]. Previous reports have shown that calcium-dependent CDPK4 and CDPK5 regulate ROS production by phosphorylating NADPH oxidase in potatoes [28]. ROS are important not only for signalling mechanisms for defence [29] but also for regulating programmed cell death via the establishment of the HR [30]. MAPK family members can improve resistance to Verticillium wilt in cotton [31]. In this study, 24 h after CEF–082 inoculation, certain signal transduction pathways may have been involved in the plant response to CEF–082 (Fig. 11). After inoculation with CEF–082, FLS2 recognized CEF–082, MAPK signal transduction was induced, and calcium channels were opened. Then, H_2O_2 was produced, leading to 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. 12). *C4H* and *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. [32], who indicated that *C4H–1* and C*4H–3* were upregulated after treatment with *V. dahliae.* Three days after inoculation with *V. dahliae,* lignin was detected, and the pith diameter of CEF–082 + *V. dahliae*-treated plants was slightly larger than that of water + *V. dahliae*-treated plants (Fig. S3). The defence response at T12h and T48h was similar to that at T0h, and only some key points induced were different in the pathways shown in Fig. 11 and Fig. 12. Thus, it is speculated that CEF–082 can control cotton Verticillium wilt because inoculation with CEF–082 can prime signalling pathways to defend against *V. dahliae* upon its infection.

When pathogens infect plants, they induce a series of defence responses. GST participates in plant defences and can remove ROS [34]. 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) [35]. *GSTF8* was used as a marker in early stress and defence responses [36], and salicylic acid, methyl jasmonate, ABA and H_2O_2 can induce *GST* expression [37–39]. *LrGSTU5* was obviously upregulated after treatment with *Fusarium oxysporum [*40], and the *GST* genes were also upregulated in *G. barbadense* treated with *V. dahliae* [41]. 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. [40–41]. 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* enhanced resistance against Verticillium wilt in tobacco [42]. Hence, we suggest that CEF–082 can induce specific *GST* genes to protect cotton from *V. dahliae.*

V. dahliae can induce a defence 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* had significantly upregulated or downregulated expression levels of resistance-related genes. Therefore, it is speculated that the defence 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 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.

Conclusion

CEF–082 can induce defensive responses in cotton, and pretreated with CEF–082 with appropriate concentration 10^5CFU/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. In this study, the transcriptome was used to study the expression of genes in cotton when CEF–82 and *V. dahliae* coexisted, which provided a basis for understanding the mechanism by which biocontrol fungi prevent Verticillium wilt in cotton.

Abbreviations

FLS2: LRR receptor-like serine/threonine-protein kinase FLS2; Rboh: respiratory burst oxidase; CDPK: calcium-dependent protein kinase. CYP: Cytochrome P450 proteins.

Declarations

Acknowledgements

We thank members of Professor Zhu’s groups for technical assistance, financial support and helpful discussions.

Funding

This work was supported by National Key Research and Development Program of China (2017YFD0201900) and National Natural Science Foundation of China (31901938).We thank all the foundation of economic support. The funding organizations provided the financial support to the research projects, and involved in the design of the study.

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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Tables

Table 1 KEGG Pathway enrichment of 5638 DEGs

Pathway ID	Pathway	Number of DEGs	*P*-value	Q-value
ko04626	Plant-pathogen interaction	376	2.57E-51	3.47E-49
ko04016	MAPK signalling pathway-plant	304	2.77E-25	1.87E-23
ko00941	Flavonoid biosynthesis	57	4.37E-08	1.97E-06
ko00940	Phenylpropanoid biosynthesis	135	1.80E-07	6.06E-06
ko00052	Galactose metabolism	88	6.14E-06	0.000166
ko00590	Arachidonic acid metabolism	34	2.09E-05	0.000469
ko00906	Carotenoid biosynthesis	39	0.000109	0.002102
ko00480	Glutathione metabolism	68	0.000138	0.002331
ko00909	Sesquiterpenoid and triterpenoid biosynthesis	21	0.000332	0.004976
ko00591	Linoleic acid metabolism	22	0.000788	0.010643
ko00511	Other glycan degradation	42	0.001074	0.013183
ko00604	Glycosphingolipid biosynthesis - ganglio series	27	0.001366	0.015371
ko00905	Brassinosteroid biosynthesis	15	0.001554	0.016135
ko00904	Diterpenoid biosynthesis	36	0.003500	0.033752
ko00600	Sphingolipid metabolism	44	0.004324	0.038919

Pathways with a Q-value < 0.05 are shown.

Table 2 GO enrichment of 5638 DEGs

Term ID	Term	Number of DEGs	*P*-value	Q-value
GO:0031224	intrinsic component of membrane	1494	2.76E-26	6.24E-23
GO:0016021	integral component of membrane	1486	6.55E-25	7.42E-22
GO:0030246	carbohydrate binding	141	6.90E-22	5.20E-19
GO:0044425	membrane part	1506	2.47E-20	1.40E-17
GO:0009607	response to biotic stimulus	60	1.13E-18	5.14E-16
GO:0016020	membrane	1542	4.17E-18	1.57E-15
GO:0005576	extracellular region	152	8.49E-18	2.74E-15
GO:0001871	pattern binding	60	2.23E-17	5.60E-15
GO:0030247	polysaccharide binding	60	2.23E-17	5.60E-15
GO:0005618	cell wall	74	1.50E-14	3.08E-12
GO:0030312	external encapsulating structure	74	1.50E-14	3.08E-12
GO:0003824	catalytic activity	2165	3.11E-14	5.86E-12
GO:0051704	multi-organism process	59	5.08E-14	8.84E-12
GO:0044036	cell wall macromolecule metabolic process	45	9.14E-14	1.48E-11
GO:0071554	cell wall organization or biogenesis	119	1.43E-13	2.16E-11

Terms with a Q-value < 0.001 are shown.

Table 3 KEGG pathway enrichment of 463 DEGs

Pathway ID	Pathway	Number of DEGs	*P*-value	Q-value
ko00940	Phenylpropanoid biosynthesis	23	4.25E-06	0.000304
ko00941	Flavonoid biosynthesis	12	6.33E-06	0.000304
ko00052	Galactose metabolism	17	1.06E-05	0.000339
ko04626	Plant-pathogen interaction	29	0.000725	0.011595
ko00232	Caffeine metabolism	3	0.001058	0.014514
ko00909	Sesquiterpenoid and triterpenoid biosynthesis	5	0.001558	0.018699

Pathways with corrected-*p* (Q-value) < 0.05 are shown.

Table 4 KEGG pathway enrichment of 1209 DEGs

Pathway ID	Pathway	Number of DEGs	*P*-value	Q-value
ko00941	Flavonoid biosynthesis	19	0.000016	0.002007
ko00901	Indole alkaloid biosynthesis	14	0.000042	0.002610
ko04016	MAPK signalling pathway - plant	60	0.000474	0.019427
ko04626	Plant-pathogen interaction	59	0.001099	0.033788
ko00940	Phenylpropanoid biosynthesis	34	0.001932	0.047538

Pathways with corrected-*p* (Q-value) < 0.05 are shown.

Table 5 KEGG Pathway enrichment of 96 DEGs

Pathway ID	Pathway	Number of DEGs	*P*-value	Q-value
ko00480	Glutathione metabolism	5	0.001184	0.035893
ko00941	Flavonoid biosynthesis	4	0.001496	0.035893

Pathways with a *Q*-value < 0.05 are shown.

Table 6-1 Predicted R genes induced by CEF-082 and *V. dahliae*

Transcript ID	Type	log2-Fold Change (12 h)	log2-Fold Change (48 h)	Nr Functional Annotation
Gh_A01G0315	CNL	1.037589939	1.4113336	disease resistance protein At4g27190-like, partial [*Gossypium hirsutum*]
Gh_A08G1253	CNL	-6.740709401	5.746950454	kelch repeat type 1 [*Corchorus capsularis*]
Gh_D02G0329	CNL	-5.561290274	4.673143293	probable glutathione S-transferase [*Gossypium hirsutum*]
Gh_D07G2361	CNL	1.502212669	-4.237409783	hypothetical protein B456_001G187600 [*Gossypium raimondii*]
Gh_D11G2274	CNL	-1.125013164	-1.176243053	AAA-ATPase At1g43910-like [*Gossypium hirsutum*]
Gh_A04G0855	CN	5.289214165	4.734256842	uncharacterized protein LOC108457923 isoform X2 [*Gossypium arboreum*]
Gh_D09G1718	CN	1.293119787	1.144955552	uncharacterized protein LOC105800125 [*Gossypium raimondii*]
Gh_Sca089655G01	CN	-1.657997189	-1.535872284	uncharacterized protein LOC107949870 [*Gossypium hirsutum*]
Gh_A06G1937	Mlo-like	1.358973461	1.345179474	MLO-like protein 2 [*Gossypium hirsutum*]
Gh_A01G2142	NL	-1.561586712	-1.020990318	NAC domain-containing protein 90-like [*Gossypium arboreum*]
Gh_A09G1261	NL	2.484985295	2.969406864	E3 ubiquitin-protein ligase PUB23-like [*Gossypium hirsutum*]
Gh_A09G1326	NL	-1.010963189	1.6799656	B3 domain-containing protein At2g36080-like [*Gossypium arboreum*]
Gh_D08G1656	NL	-5.969729954	-5.677096641	MOB kinase activator-like 1A isoform X1 [*Gossypium hirsutum*]
Gh_D11G3107	NL	3.221799962	-4.647807823	uncharacterized protein LOC107925949 [*Gossypium hirsutum*]
Gh_A01G0470	N	8.54387182	10.19853857	putative RING-H2 finger protein ATL19 [*Gossypium hirsutum*]
Gh_A03G1126	N	1.517931636	1.090903245	putative ABC transporter C family member 15 [*Theobroma cacao*]
Gh_A07G1963	N	1.113205106	1.574240958	ABC transporter B family member 19-like isoform X1 [*Gossypium hirsutum*]
Gh_D09G0181	N	-1.254590611	1.59727426	ABC transporter B family member 19 [*Gossypium arboreum*]
Gh_D09G1048	N	-1.254965579	1.436429867	ABC transporter G family member 23-like [*Gossypium hirsutum*]
Gh_D11G0790	N	6.846798953	7.786264797	putative casein kinase II subunit beta-4 [*Gossypium hirsutum*]
Gh_D11G3289	N	-3.221611506	1.159392828	ABC transporter G family member 20-like [*Gossypium hirsutum*]
Gh_A01G0355	RLP	1.257686713	1.389731263	LRR receptor-like serine/threonine-protein kinase GSO1 [*Gossypium arboreum*]
Gh_D01G0066	RLP	2.472710792	-1.905542187	hypothetical protein B456_002G007800 [*Gossypium raimondii*]
Gh_D01G0386	RLP	1.338494948	1.470239195	probable LRR receptor-like serine/threonine-protein kinase At3g47570 isoform X1 [*Gossypium hirsutum*]

Table 6-2 Predicted R genes induced by CEF-082 and *V. dahliae*

Transcript ID	Type	log2-Fold Change (12 h)	log2-Fold Change (48 h)	Nr Functional Annotation
Gh_D05G3613	RLP	-3.636894741	-1.070068403	flavonol sulfotransferase-like [*Gossypium hirsutum*]
Gh_D05G3615	RLP	3.336220891	2.121111678	hypothetical protein B456_009G443300 [*Gossypium raimondii*]
Gh_D05G3699	RLP	3.056269729	4.673541612	kinesin KP1-like [*Gossypium raimondii*]
Gh_D08G1871	RLP	1.08210341	1.06909527	probable LRR receptor-like serine/threonine-protein kinase At1g34110 [*Gossypium hirsutum*]
Gh_A10G2072	TNL	1.586976576	1.014417675	TMV resistance protein N-like [*Gossypium hirsutum*]
Gh_A11G2091	TNL	1.199688858	1.260578514	transcription repressor MYB5-like [*Gossypium arboreum*]
Gh_D01G0539	TNL	-3.861003635	1.878860421	MYB-related protein 330 [*Gossypium hirsutum*]
Gh_D01G1550	TNL	1.22796154	1.351072869	lipase [*Corchorus capsularis*]
Gh_D07G2090	TNL	-1.58798514	-1.255994867	MYB transcription factor MYB30 [*Gossypium hirsutum*]
Gh_D08G0256	TNL	-3.281882012	1.421995876	transcription repressor MYB6-like [*Gossypium hirsutum*]
Gh_D09G1659	TNL	2.803942312	1.245838921	MYB-related protein 308-like [*Gossypium hirsutum*]
Gh_D10G2351	TNL	6.829614248	1.155493756	TMV resistance protein N-like isoform X2 [*Gossypium hirsutum*]
Gh_D11G0336	TNL	1.925007761	1.072893447	MYB-related protein 306 [*Gossypium hirsutum*]
Gh_A06G1144	T	1.922712186	1.745773435	ethylene-responsive transcription factor 4-like [*Gossypium hirsutum*]
Gh_A12G1620	T	-1.404115121	1.637338509	NAC domain-containing protein 100-like [*Gossypium hirsutum*]
Gh_D01G0514	T	1.231975072	1.260531662	NAC domain-containing protein 72-like [*Gossypium hirsutum*]
Gh_D06G1403	T	1.291258193	1.247681127	ethylene-responsive transcription factor 4-like [*Gossypium hirsutum*]
Gh_D10G1537	T	2.276313579	4.332088904	ethylene-responsive transcription factor 1B-like [*Gossypium raimondii*]
Gh_D12G2494	T	-1.419500468	1.382825786	putative dehydration responsive element binding protein [*Gossypium hirsutum*]
Gh_A03G2044	Other	1.343265394	-1.171383404	thaumatin-like protein [*Gossypium arboreum*]
Gh_D11G2998	Other	2.626616578	-1.757148487	thaumatin-like protein isoform X1 [*Gossypium hirsutum*]
Gh_A02G0236	/	2.076793222	1.040351538	chalcone synthase [*Vaccinium ashei*]
Gh_A04G0830	/	10.27309684	7.911807354	glutathione S-transferase U16-like [Gossypium hirsutum]

Table 6-3 Predicted R genes induced by CEF-082 and *V. dahliae*

Transcript ID	Type	log2-Fold Change (12 h)	log2-Fold Change (48 h)	Nr Functional Annotation
Gh_A05G0560	/	0.753709773	1.881639236	DNA-damage-repair/toleration protein DRT100-like [*Gossypium raimondii*]
Gh_A05G1020	/	1.557304461	1.177105999	CBL-interacting serine/threonine-protein kinase 25-like [*Gossypium hirsutum*]
Gh_A05G3196	/	1.49233142	3.400801654	cyclic nucleotide-gated ion channel 2-like [*Gossypium arboreum*]
Gh_A05G3470	/	-1.679248485	3.0042548	NADPH:quinone oxidoreductase-like [*Gossypium arboreum*]
Gh_A06G1701	/	1.11748676	1.125924709	shikimate O-hydroxycinnamoyltransferase-like [*Gossypium arboreum*]
Gh_A09G1415	/	2.763055805	1.138512634	peroxidase 21-like [*Gossypium hirsutum*]
Gh_A11G0631	/	-1.427211837	1.313850285	probable calcium-binding protein CML45 [*Gossypium hirsutum*]
Gh_A11G1367	/	-2.131571879	-1.430486114	uncharacterized protein LOC105803388 [*Gossypium raimondii*]
Gh_A11G3297	/	4.769980778	-6.621901362	uncharacterized protein LOC107935227 [*Gossypium hirsutum*]
Gh_D02G0258	/	-2.480311701	-1.300029512	receptor-like protein 12 [*Gossypium hirsutum*]
Gh_D04G0409	/	8.681445478	5.183990265	cyclic nucleotide-gated ion channel 2-like [*Gossypium hirsutum*]
Gh_D05G0689	/	0.530244279	2.104072295	DNA-damage-repair/toleration protein DRT100 [*Theobroma cacao*]
Gh_D07G1197	/	2.197263059	1.546938795	flavonoid 3',5'-hydroxylase 2-like [*Gossypium hirsutum*]
Gh_D08G1512	/	1.61343892	2.05940005	hypothetical protein B456_002G144600 [*Gossypium raimondii*]
Gh_D09G0858	/	-1.30017514	-1.614570284	hypothetical protein B456_006G104900, partial [*Gossypium raimondii*]
Gh_D10G1431	/	1.879317791	1.074465933	chalcone synthase [*Kandelia candel*]
Gh_D11G0741	/	-1.323988617	1.425564743	probable calcium-binding protein CML45 [*Gossypium hirsutum*]
Gh_D11G1512	/	-1.351190116	-1.111728188	uncharacterized protein LOC105803388 [*Gossypium raimondii*]
Gh_D11G3107	/	3.221799962	-4.647807823	uncharacterized protein LOC107925949 [*Gossypium hirsutum*]

Additional Files Legends

Additional files 1: **Fig. S1** Correlation thermograms of the 18 samples. (DOCX 506 kb)

Additional files 2: **Fig. S2** GO enrichment of 1209 DEGs. (DOCX 54 kb)

Additional files 3: **Fig. S3** Histochemical analysis of lignin in stem cross-sections of cotton plants. (DOCX 466 kb)

Additional files 4: **Table S1** Specific primer sequences of qRT-PCR-related genes. (DOCX 15 kb)

Additional files 5: **Table S2** Sequencing quality statistics table. (DOCX 15 kb)

Additional files 6: **Table S3** Overview of novel transcripts. (DOCX 14 kb)

Additional files 7: **Table S4** Putative R genes and TFs in 96 DEGs. (DOCX 15 kb)

Additional files 8: Data of the content of H_2O_2. (XLSX 13 kb)

Additional files 9: Data of disease index. (XLSX 10 kb)

Additional files 10: Data of qPCR. (XLSX 36 kb)

Figures

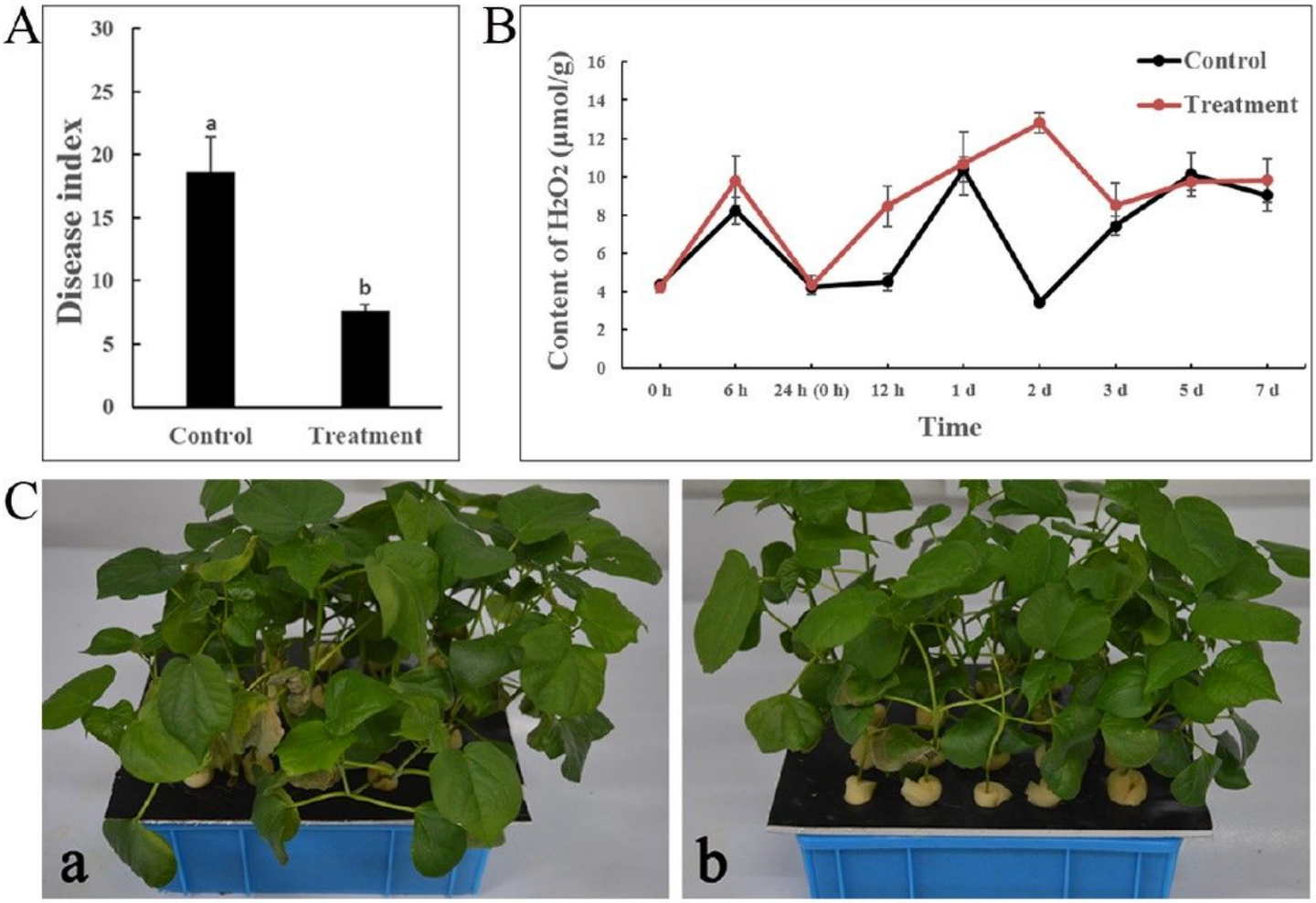

Figure 1

Disease index and symptoms of Verticillium wilt in cotton 14 d after V. dahliae inoculation. (A) The disease index. (B) The content of H2O2. (C) Symptoms of Verticillium wilt in cotton：a: water+V. dahliae, b: CEF-082+V. dahliae. Bars represent SEs.

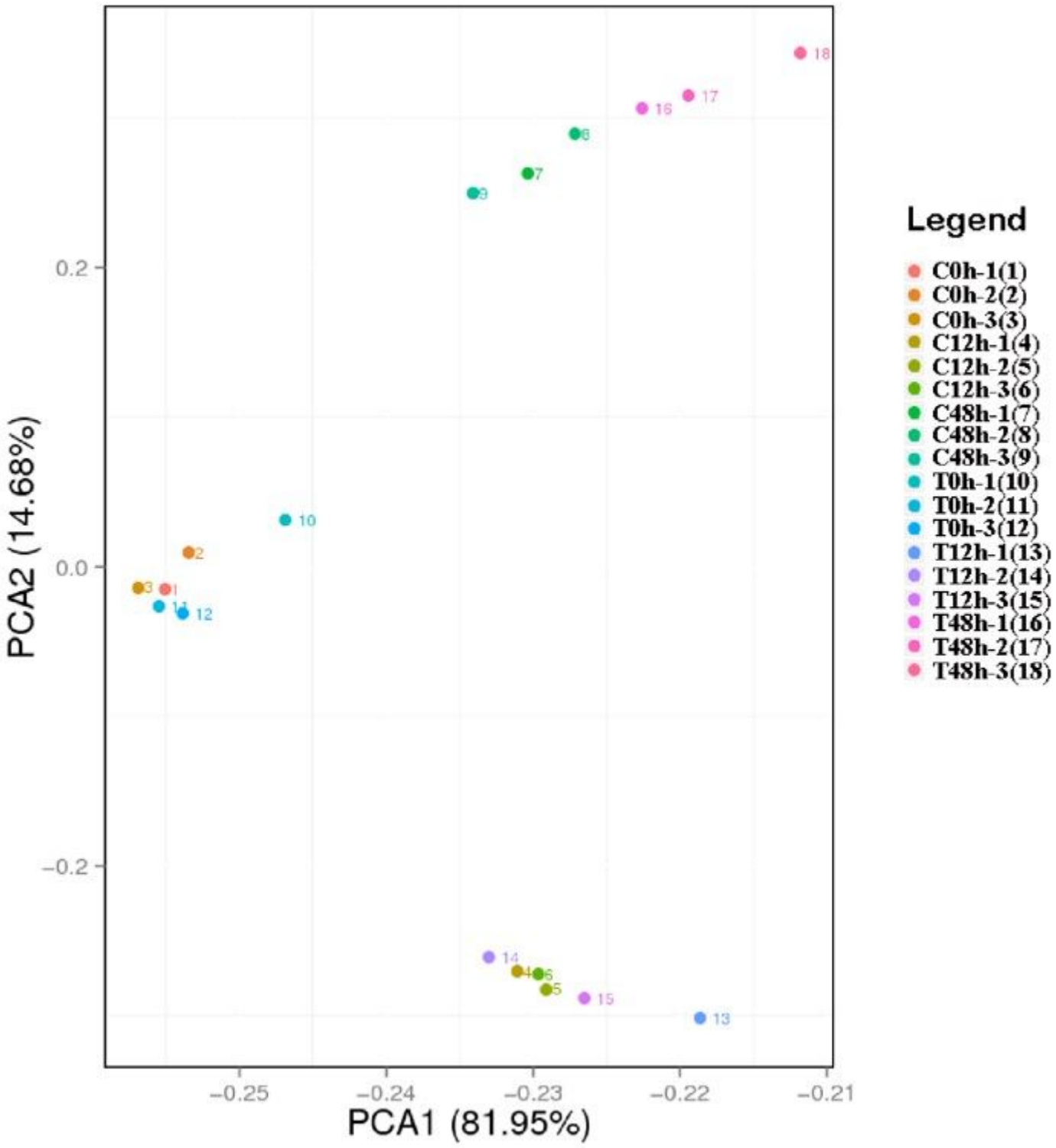

Figure 2

PCA of the 18 samples.

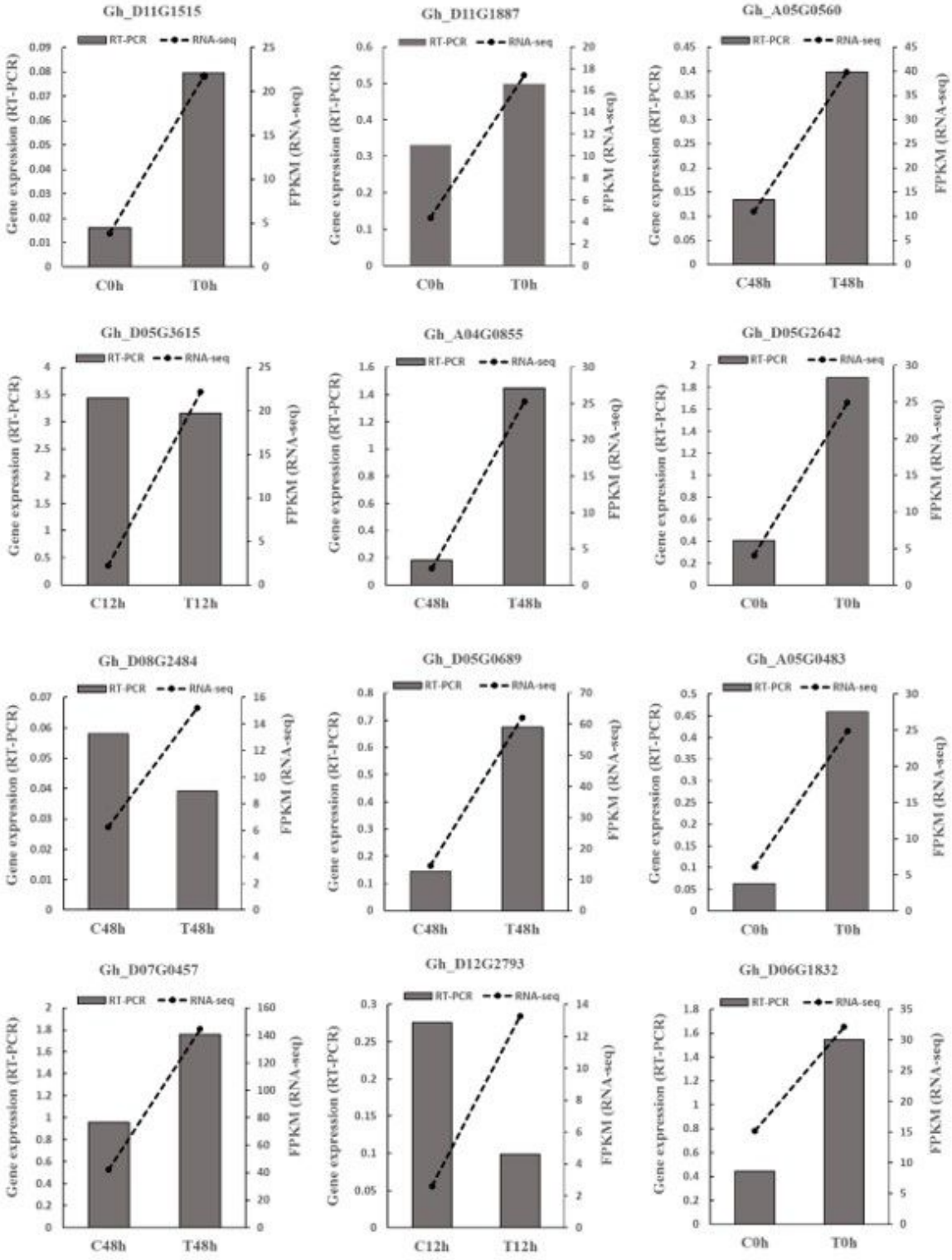

Figure 3

Comparison of the expression trends of the qRT-PCR and RNA-seq data. The grey bars represent the genes expression level relative to cotton ubiquitin gene, as an internal control, was used to normalize the expression levels of the target genes. Dotted lines represent the mean of FPKM.

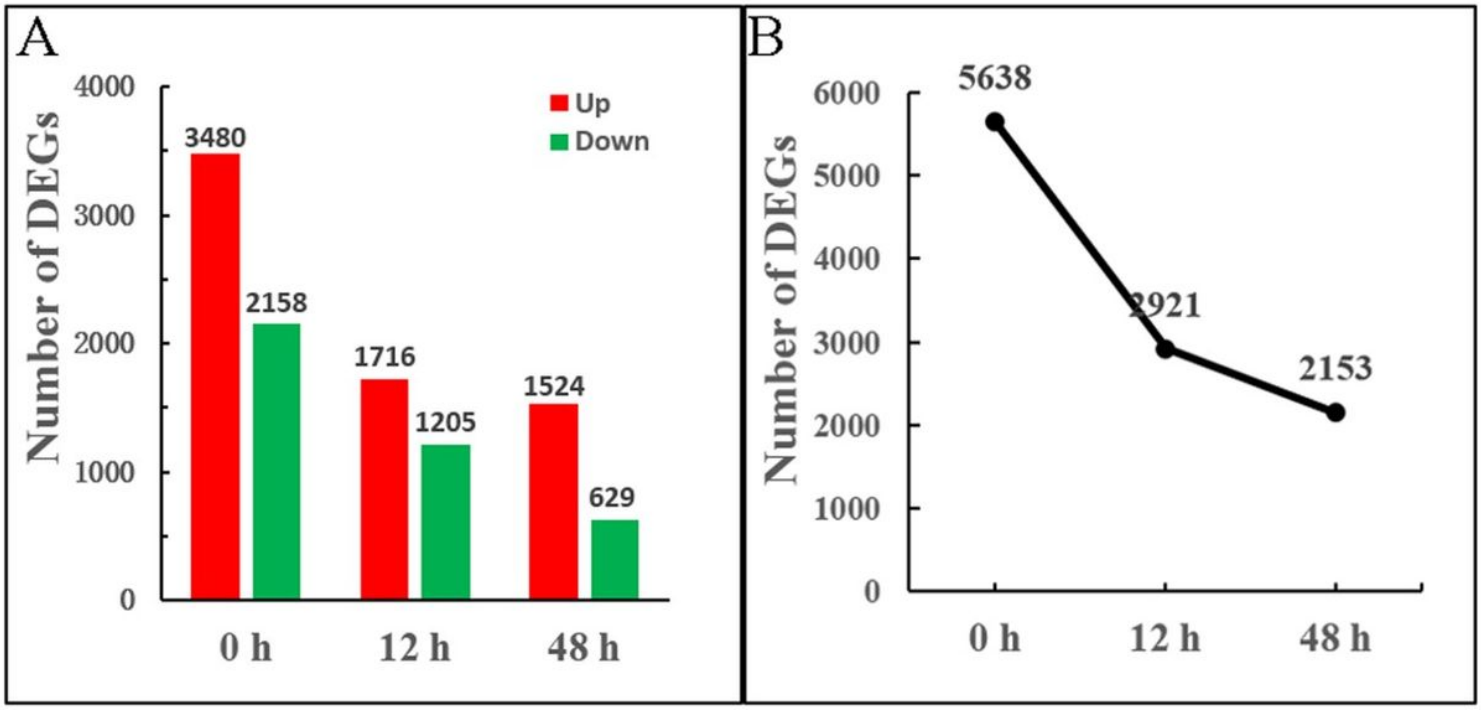

Figure 4

Number of DEGs at 0 h, 12 h, and 48 h. (A) Quantity of up- and downregulated genes at the three time points. (B) Total number of DEGs at the three time points.

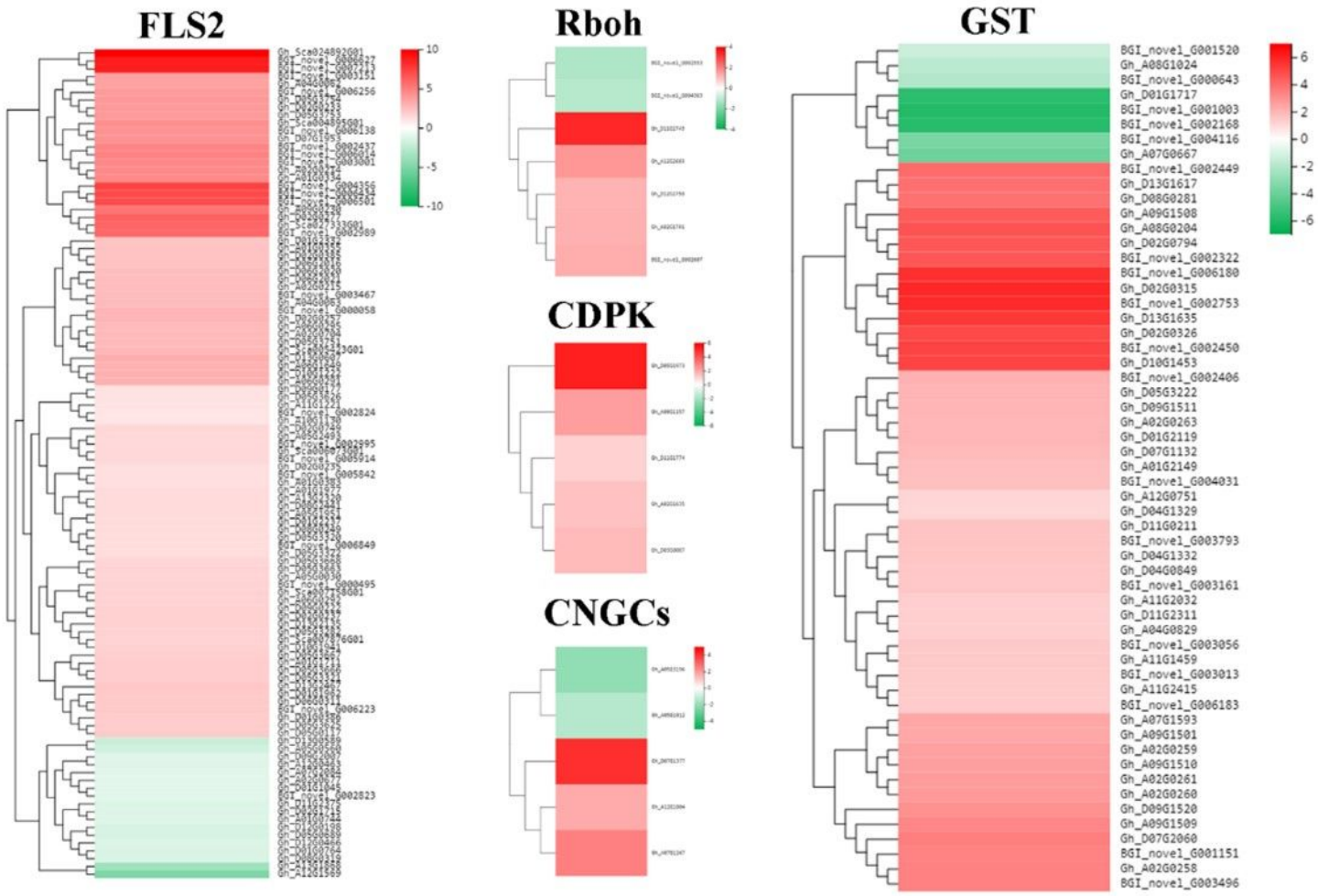

Figure 5

Expression of genes related to ROS and Ca2+. Red represents upregulation, and green represents downregulation.

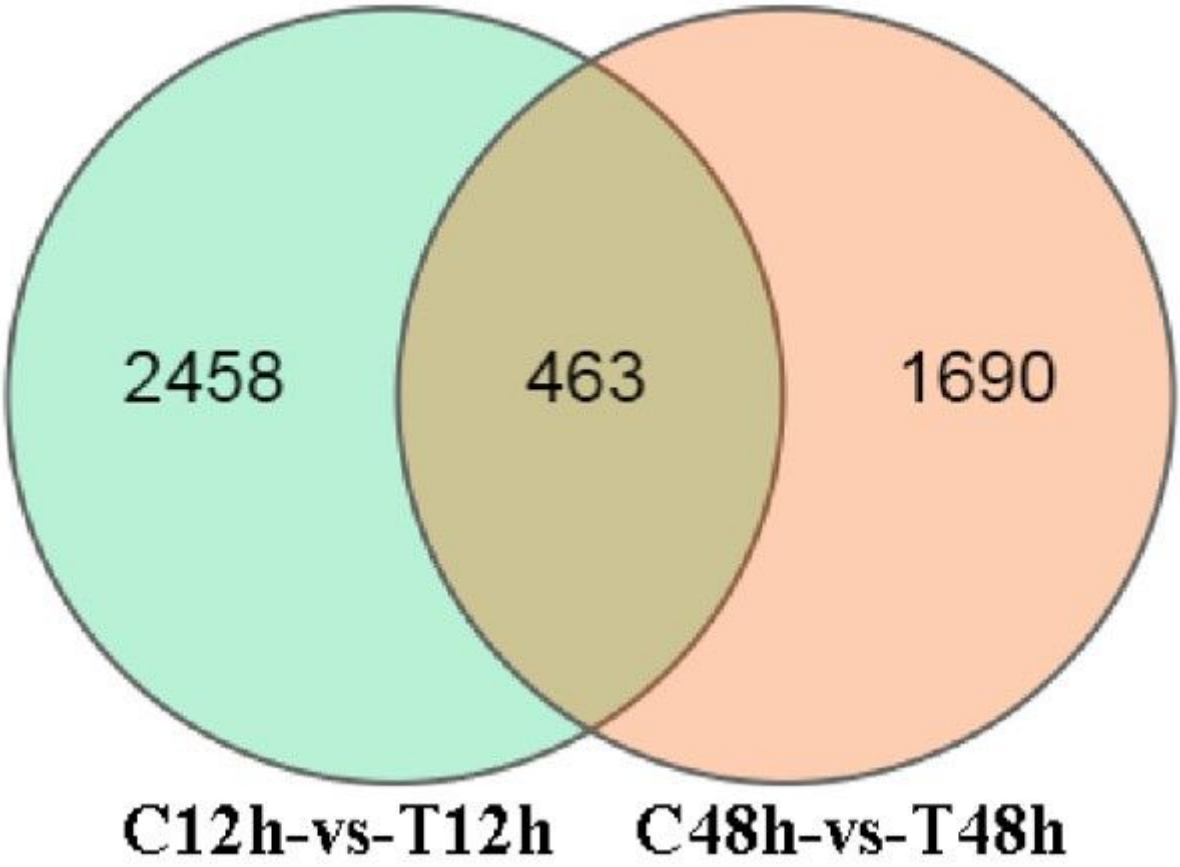

Figure 6

The distribution of DEGs 12 h and 48 h post-inoculation with V. dahliae.

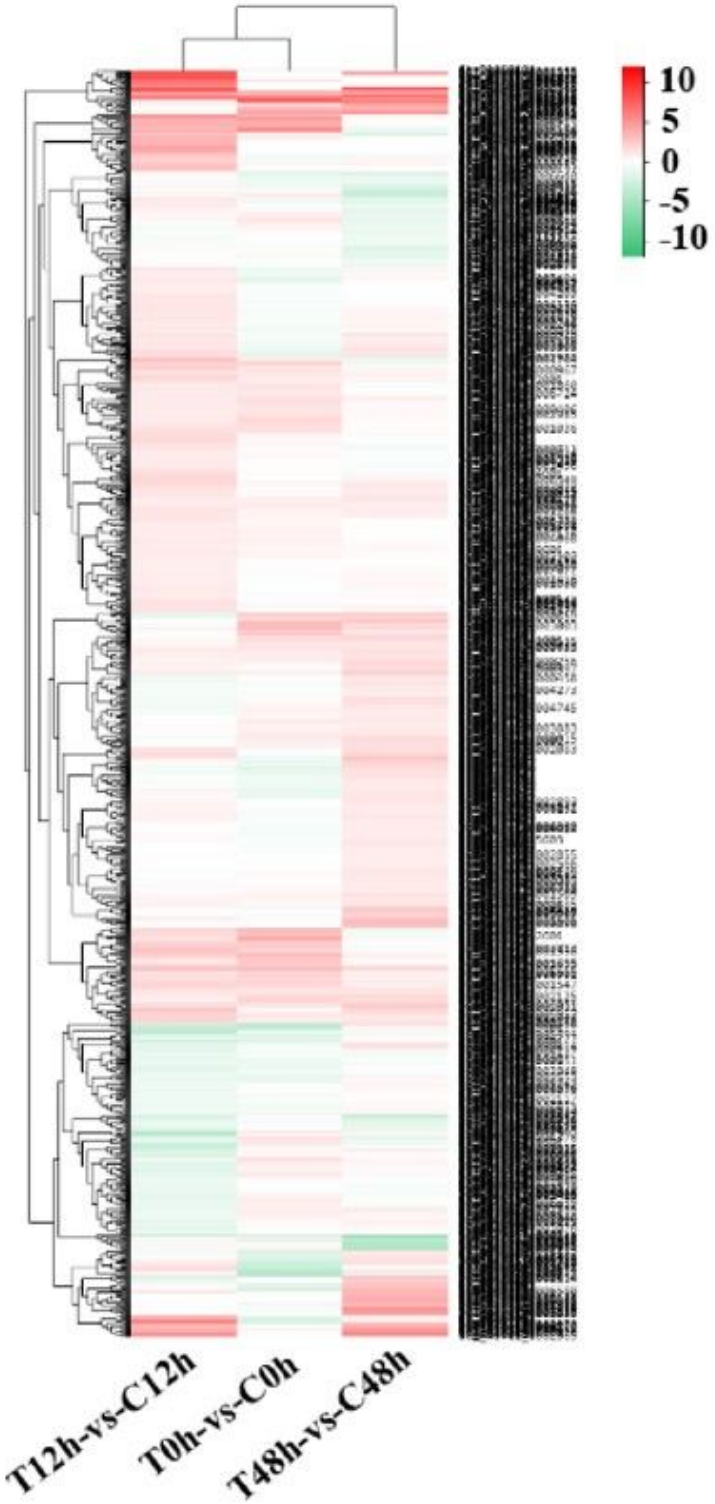

Figure 7

The clustering thermogram of 1209 DEGs.

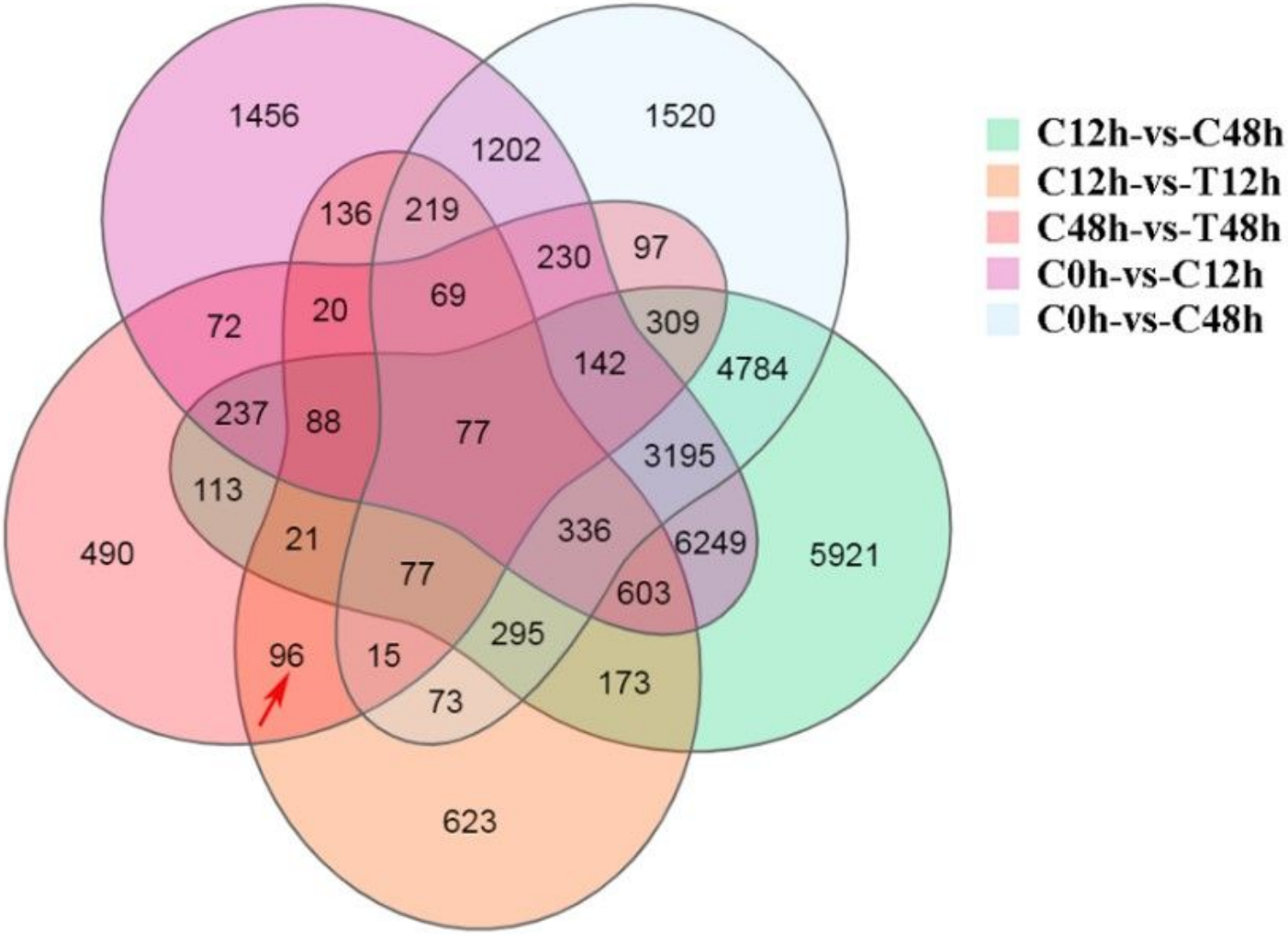

Figure 8

Venn diagram of DEGs.

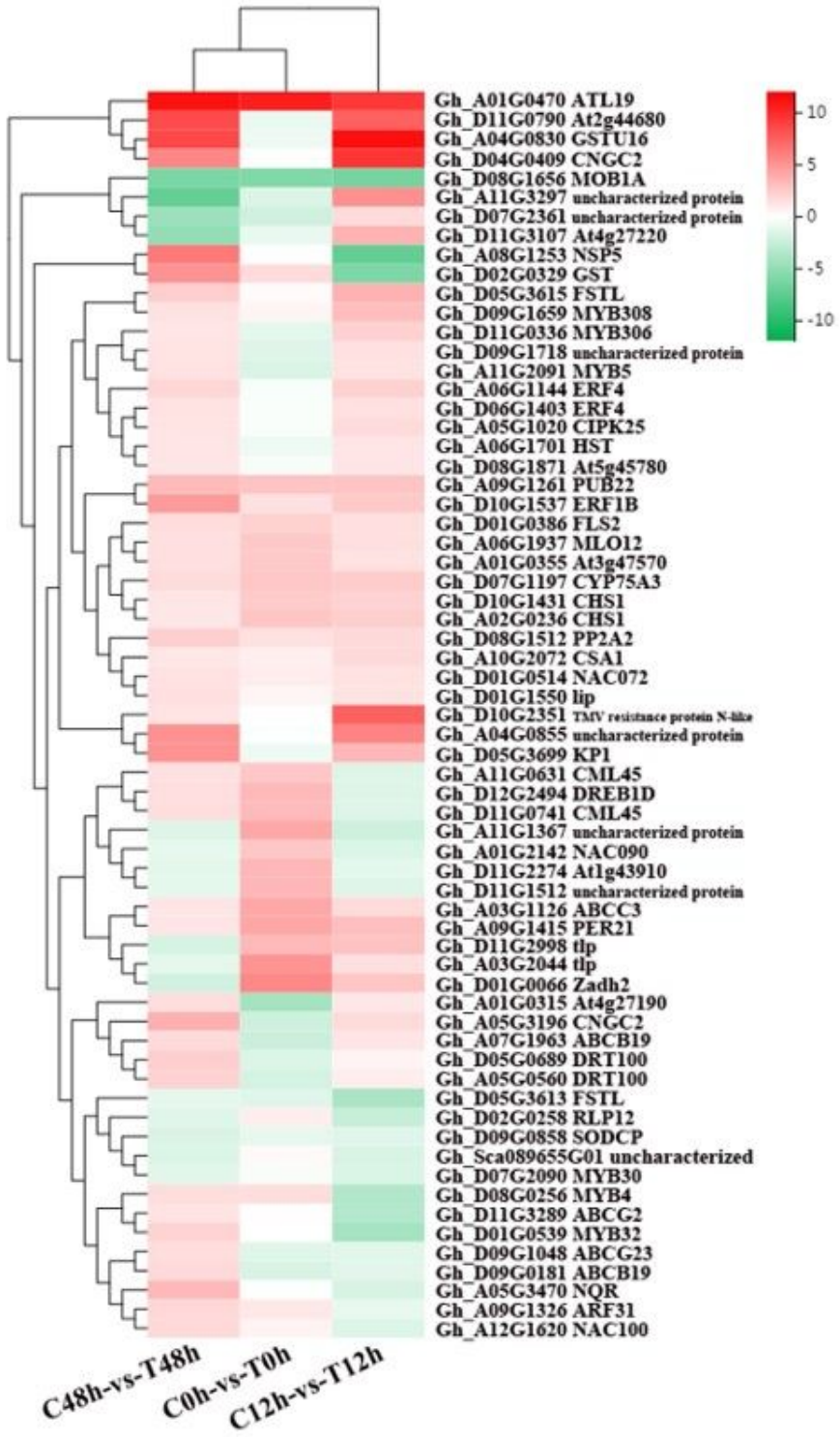

Figure 9

Clustering thermogram of putative R genes and TFs.

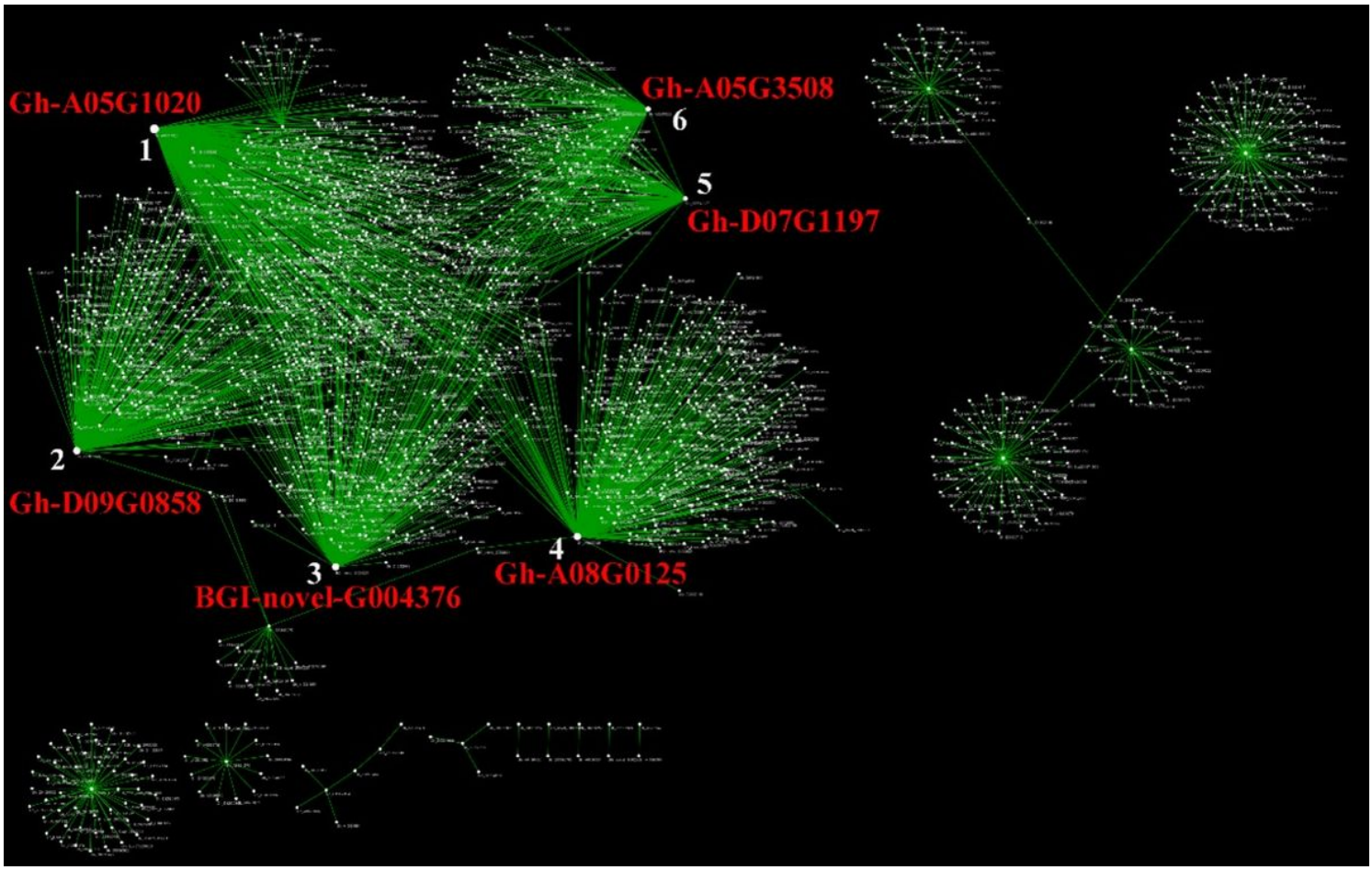

Figure 10

Protein interaction network of 96 DEGs and their related genes in cotton. The red font indicates hub genes.

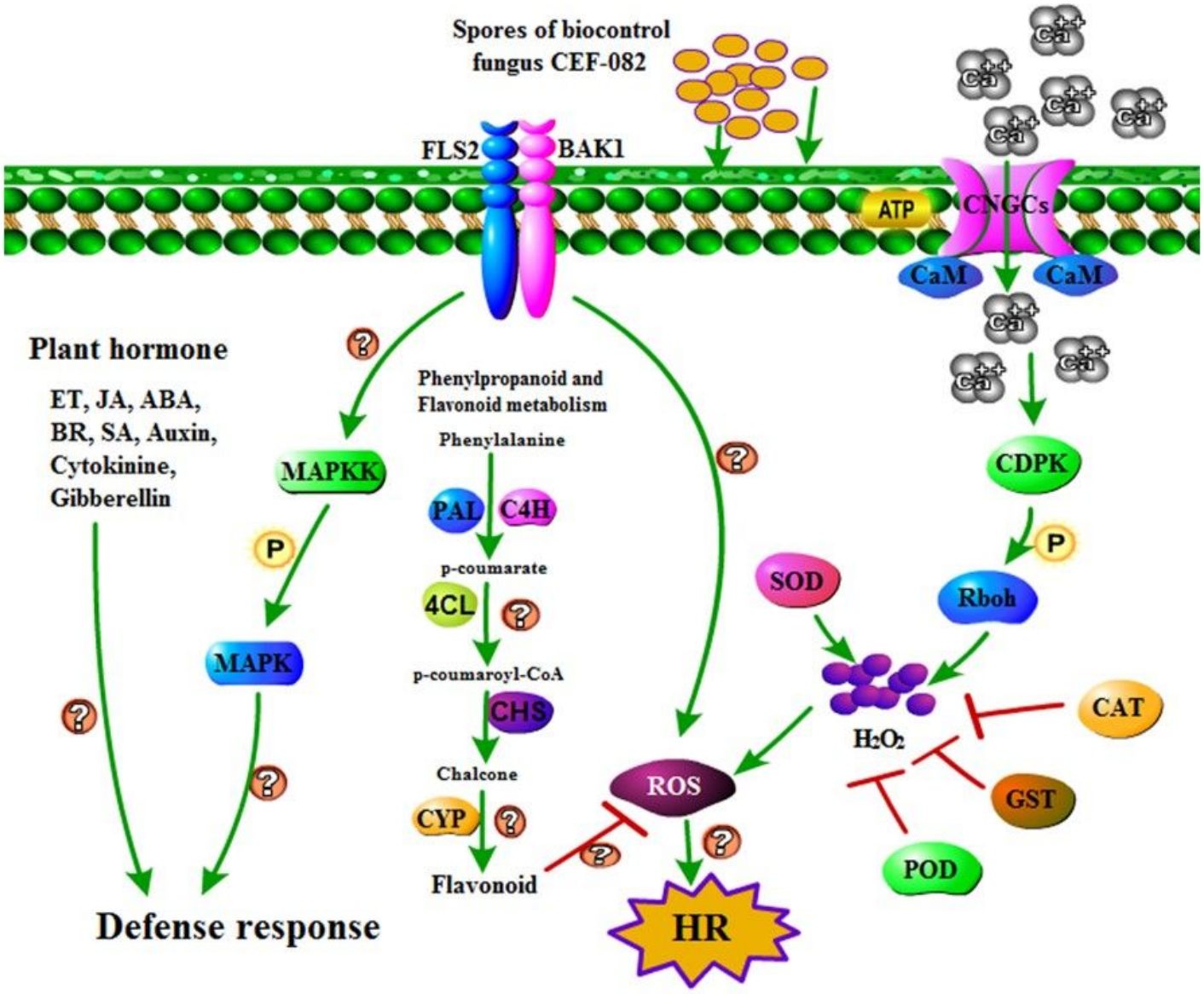

Figure 11

Signal transduction pathways induced by CEF-082.

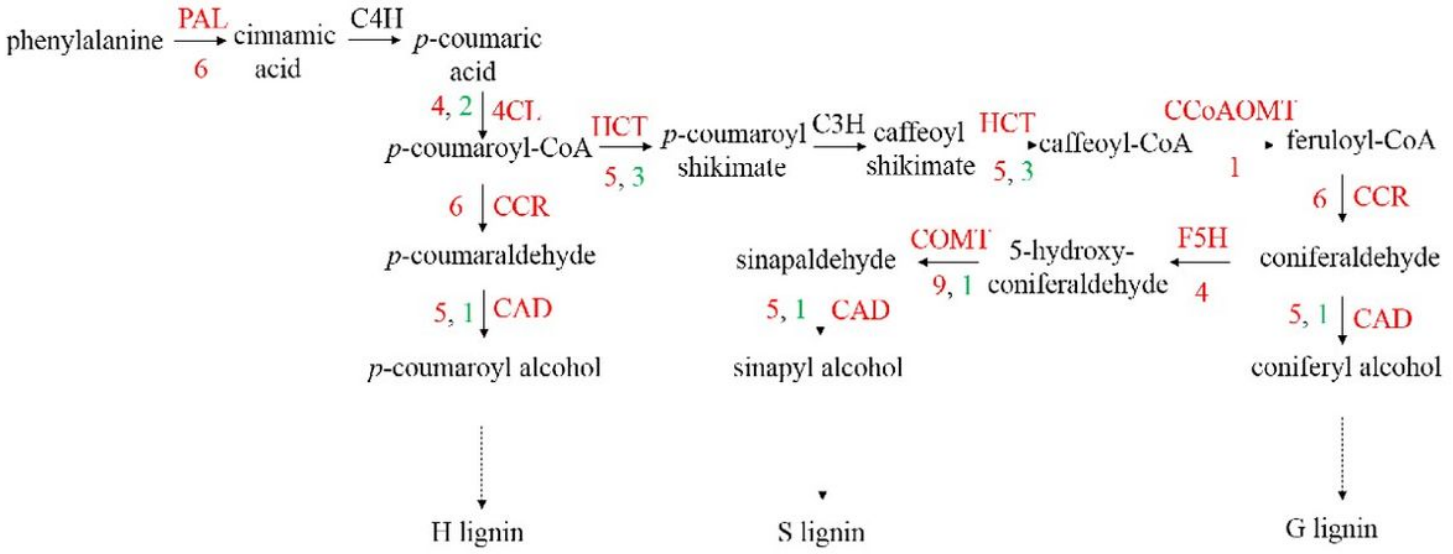

Figure 12

Lignin biosynthesis pathway [33]. Enzymes coloured in red or black indicate the key pointsinduced or uninduced by CEF-082. The red numbers represents the number of upregulated genes, and green numbers represent the number of downregulated genes. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; C3H, p-coumarate 3 hydroxylase; HCT, hydroxycinnamoyl transferase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA O-methyltransferase; F5H, ferulate-5-hydroxylase.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfiles6.docx
- Additionalfiles5.docx
- Additionalfiles4.docx
- Additionalfiles10.xlsx
- Additionalfiles9.xlsx
- Additionalfiles7.docx
- Additionalfiles2.docx
- Additionalfiles1.docx
- Additionalfiles3.docx
- Additionalfiles8.xlsx