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Research Article

The necrotroph *Botrytis cinerea* promotes disease development in *Panax ginseng* by manipulating plant defense signals and antifungal metabolites degradation

Huchen Chen, Shuhan Zhang, Shengnan He, Runa A, Mingyang Wang, Shouan Liu*

Laboratory of Tea and Medicinal Plant Pathology, Jilin University, Changchun, China

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ABSTRACT

Background: *Panax ginseng* Meyer is one of the most valuable medicinal plants which is enriched in antimicrobe secondary metabolites and widely used in traditional medicine. *Botrytis cinerea* is a necrotrophic fungus that causes gray mold disease in a broad range of hosts. *B. cinerea* could overcome the ginseng defense and cause serious leaf and root diseases with unknown mechanism.
Methods: We conducted simultaneous transcriptomic and metabolomic analysis of the host to investigate the defense response of ginseng affected by *B. cinerea*. The gene deletion and replacement were then performed to study the pathogenic gene in *B. cinerea* during ginseng - fungi interaction.
Results: Upon *B. cinerea* infection, ginseng defense responses were switched from the activation to repression, thus the expression of many defense genes decreased and the biosynthesis of antifungal metabolites were reduced. Particularly, ginseng metabolites like kaempferol, quercetin and luteolin which could inhibit fungi growth were decreased after *B. cinerea* infection. *B. cinerea* quercetin dioxygenase (Qdo) involved in catalyzing flavonoids degradation and △*BcQdo* mutants showed increased substrates accumulation and reduced disease development.
Conclusion: This work indicates the flavonoids play a role in ginseng defense and *BcQdo* involves in *B. cinerea* virulence towards the *P. ginseng*. *B. cinerea* promotes disease development in ginseng by suppressing of defense related genes expression and reduction of antifungal metabolites biosynthesis.

1. Introduction

In nature, plants are continuously under biotic or abiotic stresses that compromise plant growth and development. The biotic stresses such as fungi, bacteria or oomycetes already affect global food security and endanger the human health [1–3]. Specially, the necrotrophic *Botrytis cinerea* infects more than 1400- species worldwide, ranks itself as the second most destructive fungal pathogen [4,5]. In response to microbe attack, plants have developed complex mechanisms to detect microbes and to activate defense responses [6,7]. The plant immune system consists of two interconnected branches called PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) [8–11]. However, specific recognition of necrotrophic microbes by the same mechanisms has not been well defined [3]. Plant immunity towards *B. cinerea* appears to be under complex genetic control [12].

Upon *B. cinerea* infection, a set of downstream responses in plants are induced. These include, alterations in hormonal levels, transcriptional reprograming and the changes in plant metabolites. Particularly, the induction of hormone mediated pathways and the activation of transcription factors (TFs) have been demonstrated to play important role for plant defense against pathogens [13–15]. Plant hormones like the salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are important components in modulating immune responses. SA has been traditionally associated with defense against biotrophic or hemibiotrophic pathogens, whereas JA/ET appear to be more important to necrotrophic pathogens [13]. However, the contribution of selected hormones to host immunity varies depending on the pathogen infection strategy and nutrition requirements [3]. JA signaling was also involved in the biotrophic interaction while SA/JA pathways collaborated during ETI [16,17]. In addition, thousands of genes' expression changed in the host following pathogen infection, suggesting the involvement of key

* Corresponding author. Laboratory of Tea and Medicinal Plant Pathology, Jilin University, Changchun, 130062, China.
E-mail address: shouan@jlu.edu.cn (S. Liu).

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TFs, transcriptional co-regulators, and transcriptional machinery involved in their regulation [18]. Since plant defense depends on elaborate signaling networks controlled by hormones and TFs, it is acceptable that pathogens can use the virulence effectors to target, manipulate, or interfere with these signaling pathway [19].

Ginseng gray mold, caused by *B. cinerea*, has become one of the major diseases influencing ginseng production. In the field, *B. cinerea* is known for high variation in their aggressiveness on different plant species [20]. Thus, the outcome of experiments using different plants should be carefully analyzed. However, no such information about *B. cinerea* on medicinal plants like ginseng was observed so far. Detailed molecular and comparative analysis among ginseng - *B. cinerea* interaction is becoming feasible as the genomes of *Panax ginseng* and *B. cinerea* have been sequenced, respectively [21,22].

In this study, we performed integrated analysis of the transcriptomes and metabolomes in ginseng leaves during *B. cinerea* infection. We compared the data derived from the same treatments to obtain an integrated understanding of disease development by suppressing of defense related genes expression and reduction of antifungal flavonoids biosynthesis. We obtained a gene in *B. cinerea* (Bcin08g05000) encoding quercetin dioxygenase (*BcQdo*) involved in ginseng - *B. cinerea* interaction and impaired host defense responses by catalyzing the flavone degradation. The results of this work will increase our understanding of the complexity of ginseng - *B. cinerea* interaction and will enhance efforts to identify pathogenicity or toxicity related genes in the necrotrophic microbe and the functional genes in herbal medicine defense responses.

2. Materials and methods

2.1. Plants and fungi material

2-years-old ginseng root was grown in the pots with sterilized soil under a microbe free climate chamber with the 10 h light and 14 h dark. The heathy ginseng leaves without any disease were selected for the experiments. The *B. cinerea* B05.10 was grown on PDA plate to produce the conidia spores. The spores were harvest as previously reported [23].

2.2. Incubation and sample collection

For *B. cinerea* (*Bc*) incubation, the ginseng leaves were infected with 5×10^5 spores/ml. Plants were then kept under sealed hoods with high humidity during the infection. The buffer without any spores were sprayed as untreated control (CK) and the CK plants were kept under the clean, fungi free sealed hoods. The infection was carried out in the microbe free climate chamber under a strict light (10 h/14 h, light/dark) and temperature (22 °C/20 °C) regime. All leaves were harvested at 14 h after infection. The samples (6 replicates for each treatment) collected at the same time were used for metabolites. For qPCR assay, plants were infected with *B. cinerea* and collected at different time points (12, 24, and 48 h). All the samples were repeated three times and frozen immediately in liquid N_2 and kept at −80 °C.

2.3. Library construction and RNA sequencing

Total RNA samples were prepared for Illumina sequencing. RNA isolation, purification and monitoring, and cDNA library construction and sequencing were performed as previously [24].

2.4. Mapping fragments to the genome and quantification of gene level

Clean reads were first obtained by removing the lower quality reads (i, reads containing sequencing adaptors; ii, reads containing sequencing primer; iii, nucleotide with q quality score lower than 20) from the raw data. Q20, Q30 and GC content of the clean data were then calculated. All clean data with high-quality reads were used for downstream analyses. Reference genome and gene model annotation files were downloaded from the website (http://ginsengdb.snu.ac.kr/data.php). The raw sequence data have been submitted to the NCBI Short Read Archive with accession number GSE179805. Index of the reference genome was built and paired-end clean reads were aligned to the reference by using HISAT package [25]. Finally, the FPKM of each gene was calculated based on the length and reads count mapped to the gene [26].

2.5. Analysis of differentially expressed genes (DEGs)

Differential expression analysis of all samples (CK, Bc14 h) was performed as previously [24]. The differentially expressed genes (DEG) were selected with log2 (fold change) > 1 or log2 (fold change) <-1 and with statistical significance (p value < 0.05) by R package.

2.6. GO and KEGG enrichment analysis of DEGs

Gene Ontology (GO) enrichment analysis of DEGs was implemented by the GOseq R package, in which gene length bias was corrected [27]. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource basing on large scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies [28].

2.7. Real-Time quantitative PCR

Real-Time quantitative PCR was performed as previously described [29]. Primer sequences are listed in Table S7.

2.8. Metabolite extraction and parameter setting

For metabolites extraction, 20 μl of sample was extracted with 120 μl of precooled 50% methanol buffer. The mixture was vortexed for 1 min, incubated at room temperature for about 10 min, and after centrifugation at 4000 g for 20 min, the supernatants were transferred into new 96-well plates. Pooled quality control (QC) samples were also included by combining 10 μl of each extraction mixture. All samples were analyzed by the Liquid Chromatograph Mass Spectrometer (LC-MS) system according to machine orders.

2.9. Identification and quantification of metabolite

MSConvert was used to transform LC-MS raw data into the mzXML format, which was then processed by the XCMS, CAMERA and metaX toolbox, implemented in the R software. The combined retention time (RT) and *m*/*z* data were used to identify each ion.

2.10. Constructing B. cinerea BcQdo deletion and complementation strains

To construct the *BcQdo* (Bcin08g05000) gene replacement vector, flanking sequences of the gene were PCR-amplified from the B05.10 genomic DNA and inserted into the PXEH vector [30]. The final replacement vector was transformed with B05.10 spores using an *Agrobacterium tumefaciens* AGL1 strain. Knock-out resistant

transformants were initially screened on selective media, and then confirmed by PCR and qPCR with indicated primers (Table S7). To investigate *BcQdo* complementation lines, the PCR fragment encoding the full-length of gene was isolated and cloned in-frame into the modified pCAMBIA1303 [31]. Then, the vector was transformed into△*BcQdo* spores to obtain the complement strains. For infection the indicated plants such as ginseng and soybean, at least three biological replicates each with more than three repeats were performed on intact plants. Upon completion of the infection experiments, leaves were detached and photographed.

2.11. The antifungal activity analysis

The effect of the flavonoids on *B. cinerea* growth was assessed using the mycelial plugs growth method. The compounds were mixed with sterile melting PDA medium to obtain final concentrations. The incubation of fungi, the measurement of mycelial growth, and the relative inhibition ratio were performed as previously [29,32].

2.12. Quercetin degradation by B. cinerea △BcQdo mutants

To analysis the degradation effect of BcQdo on flavonoids, the quercetin was added in CM liquid medium to a final concentration of 1.0 mg/ml and then incubated with same amounts of fresh spores of *B. cinerea* B05.10 and △*BcQdo* mutants (△*BcQdo*-6 and △*BcQdo*-12), respectively. All samples were incubated by shaking at 28 °C with 180 rpm under darkness. Samples were harvested at 0 h, 20 h and 40 h, respectively, and each with three repeats. The absorbance value of each sample was determined by sodium nitrite-aluminum nitrate colorimetric method, and the relative flavonoid content was calculated.

2.13. Statistical analysis

The data were analyzed by analysis of variance using Statistical Product and Service Solutions (SPSS) 18 software (IBM). The differences were considered significant at * $P < 0.05$, **$P < 0.01$, respectively. All the data are represented as the mean ± SEM of at least three independent experiments.

3. Results

3.1. Transcriptome analysis of differentially expressed genes in P. ginseng during plant - B. cinerea interaction

The medicinal plant *P. ginseng* faces to various pathogens during its long life. To see the response of ginseng towards *B. cinerea*, we incubated ginseng leaves with a virulent strain B05.10. Three days post incubation, the fungi grew and the lesion developed on the leaves of ginseng (Fig. 1A). It indicates the ginseng is susceptible to *B. cinerea* B05.10.

To understand the defense or the pathogenic mechanism between medicinal plant and fungal pathogen, we performed RNA sequencing to test ginseng transcripts changes. *B. cinerea* spray-inoculated ginseng leaves (14 h post infection, Bc14 h) and mock-treated ginseng leaves (control, CK) were used. Around 50 million high quality reads of about 8 G base pairs (bp) were generated for each library. A total of 310 million validated high-quality reads were obtained from all six libraries (Table S1). The reads were aligned to the *P. ginseng* genome [22,33].

To identify genes involved in ginseng response to *B. cinerea* at the genome-wide level, we compared statistically significantly differentially changed genes (altered at least two-folds, $P \leq 0.05$, SSTF) between *B. cinerea*-treated (*Bc*) and un-treated (CK) ginseng. A total of 4244 SSTF genes were identified in *Bc*-treated plants compared with CK.

Since pathogen infection often up- or down-regulation of host genes expressions during interaction, we further analyzed the SSTF genes in ginseng. As indicated in Fig. 1B and 1744 genes were up-regulated while 2500 genes were down-regulated upon *B. cinerea* infection. To our surprise, around 60% of SSTFs in ginseng are down-regulated by *B. cinerea* B05.10 infection, which are different with previous report in other plants, suggesting these genes play a special role in medicinal plant - fungus interaction. The up-regulated and down-regulated genes were additionally analyzed by GO and KEGG methods, respectively.

For the up-regulated genes, GO terms about TF activity, sequence-specific DNA binding, part of the genes response to JA are enriched (Fig. 1C; Dataset S1; Table S2; Figs S1A, S2). KEGG analysis indicated the genes associated with the plant-pathogen interaction are enriched in up-regulated genes (Fig. S1B). For the down-regulated genes, GO terms about defense responses, defense response to fungus, certain TFs, secondary metabolite biosynthetic process etc, are enriched (Figs. 1C and 2; Dataset S1; Table S2; Figs S1C, S2). KEGG analysis indicated the genes associated with the plant-pathogen interaction are also enriched in down-regulated genes (Fig. S1 D).

GO and KEGG analysis of SSTF genes in ginseng indicated that defense and metabolism biosynthesis related genes are enriched after *B. cinerea* infection, and many of them are down-regulated by this pathogen. For example, 126 genes encoding TFs are observed upon *B. cinerea* infection, while 86 of them are down-regulated compared with CK (Dataset S1). Table S2 indicates 29 SSTF genes involving in JA/ET pathway, but 21 of them are suppressed by *B. cinerea.* Since certain transcription factors and hormones play important role for plant defense against pathogens, the down-regulation of TFs and JA/ET pathway genes in ginseng by *B. cinerea* might contribute to disease development.

3.2. Metabolomics analysis of differentially changed metabolites in P. ginseng during plant - B. cinerea infection

To explore the change of metabolites in ginseng after *B. cinerea* infection, a metabolome approach was performed by LC-MS. The final statistics showed that 15,112 and 9009 metabolites were obtained by the positive (POS) and negative (NEG) models, 6874 and 2887 of which were annotated, respectively (Table S3). The metabolites identified were then assigned to the KEGG databases and several stress related metabolisms are specially enriched (Fig. S4).

To provide a deep overview of the metabolic changes during ginseng - *B. cinerea* interaction, several quality control parameters for the quantification were performed (Figs S4, S5; Table S4). Dramatic variations in the metabolomes between CK and *Bc* were shown in Fig. 3A and B. Final statistical analysis identified 969 significant differentially accumulated metabolites (DAMs) between *Bc*-infected plants and CK (Fig. 3B; Fig. S6). 366 metabolites are presented as being up-regulated while 603 are down-regulated (Fig. 3B; Table S4). All the DAMs were assigned to various major metabolic categories (Fig. 3C). For example, 16 DAMs are enriched in flavone and flavonol biosynthesis, 14 DAMs are enriched in isoflavonoid biosynthesis, 11 DAMs are involved in phenylpropanoid biosynthesis while 6 DAMs are enriched in phenylalanine metabolism (Fig. S7A-C; Table S5).

Interestingly, more than 62.0% of DAMs are decreased after *B. cinerea* infection (Fig. 3B; Table S4). For example, all identified metabolites involved in flavonoid biosynthesis and isoflavonoid biosynthesis are significantly decreased in *B. cinerea* infected plants at 14 hpi (Fig. S7B, C). In addition, 12 out of 14 metabolites in indole alkaloid biosynthesis are down-regulated by *B. cinerea.* Similarly,

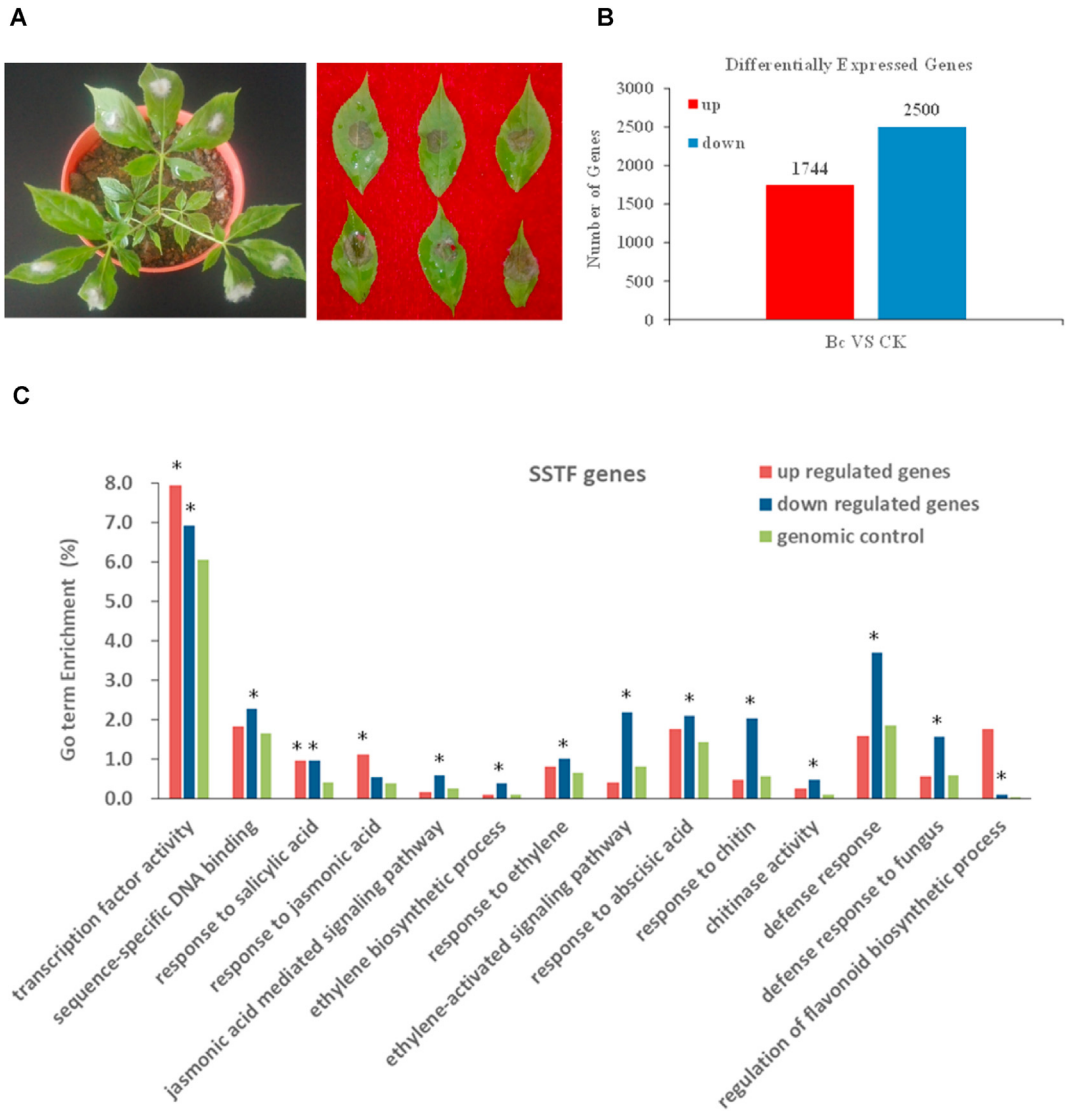

Fig. 1. Differentially expressed genes in ginseng response to *B. cinerea* B05.10. (A) *B. cinerea* B05.10 infection phenotype on 2-years old ginseng leaves (3 days post infection). (B) Numbers of differentially expressed genes (≥2-fold; $P \leq 0.05$) in ginseng at 14 h after mock treatment (CK) or spray inoculation with spores of *B. cinerea* B05.10 (*Bc*) identified by RNA-seq. (C) Gene Ontology (GO) analysis SSTFs associated with defense response, transcription factors, hormone responses, response to chitin and regulation of flavonoid biosynthesis. Numbers indicate the percentages of genes belonging to each GO category. Asterisks indicate significant enrichment ($P < 0.05$) of genes associated to the respective GO term within a gene set as determined by GO term.

most of the detected metabolites in phenylalanine metabolism and phenylpropanoid biosynthesis were suppressed by *B. cinerea* (Table S5). Except metabolism in flavonoid and isoflavonoid biosynthesis, we also observed the JA, methyl jasmonate (MeJA) and (+)-7-isomethyljasmonate were significantly decreased after *B. cinerea* infection (Fig. S8). JA and its derivates are commonly reported to play role in plant defense to *B. cinerea*.

3.3. Comprehensive analysis of metabolome and transcriptome revealed the flavonoids play a role in ginseng - B. cinerea interaction

In order to investigate the association between metabolites and genes involved in the same biological process (KEGG) pathway, the comprehensive analysis of metabolome or transcriptome was performed using Pearson's Correlation Coefficient, respectively. The results showed that 1988 SSTF genes participated in 130 pathways, while 73 differentially accumulated metabolites involved in 51 pathways (Dataset S2).

The secondary metabolites flavonoids contribute to plant environmental adaptation, fruit development, and even human health [34]. GO term "regulation of flavonoid biosynthetic process" was enriched in down-regulated genes. For example, expression of *Pg_S1550.24*, *Pg_S2797.5*, *Pg_S2242.7*, *Pg_S5852.9*, *Pg_S2473.26*, *Pg_S0171.31*, *Pg_S0447.20*, *Pg_S4060.1* and *Pg_S0753.4* was decreased at 14 h (Fig. 4). As the consequence, many of the metabolites associated with flavonoid are decreased (Dataset S3; Table S6), such as kaempferol, quercetin, luteolin, etc. The decrease of flavonoids during ginseng - *B. cinerea* interaction suggests these compounds may play a role in plant defense.

To know if the compounds have antifungal activity towards *B. cinerea*, we incubate *B. cinerea* on PDA plates with several chemicals in flavonoids (1.0 mg/ml) (Fig. 5). These flavonoids include kaempferol, quercetin, luteolin and hesperetin. The PDA plate without any chemical was used as control (CK). The kaempferol, quercetin and luteolin could inhibit fungal growth since the colony sizes were smaller than CK at 24 h post incubation (Fig. 5A).

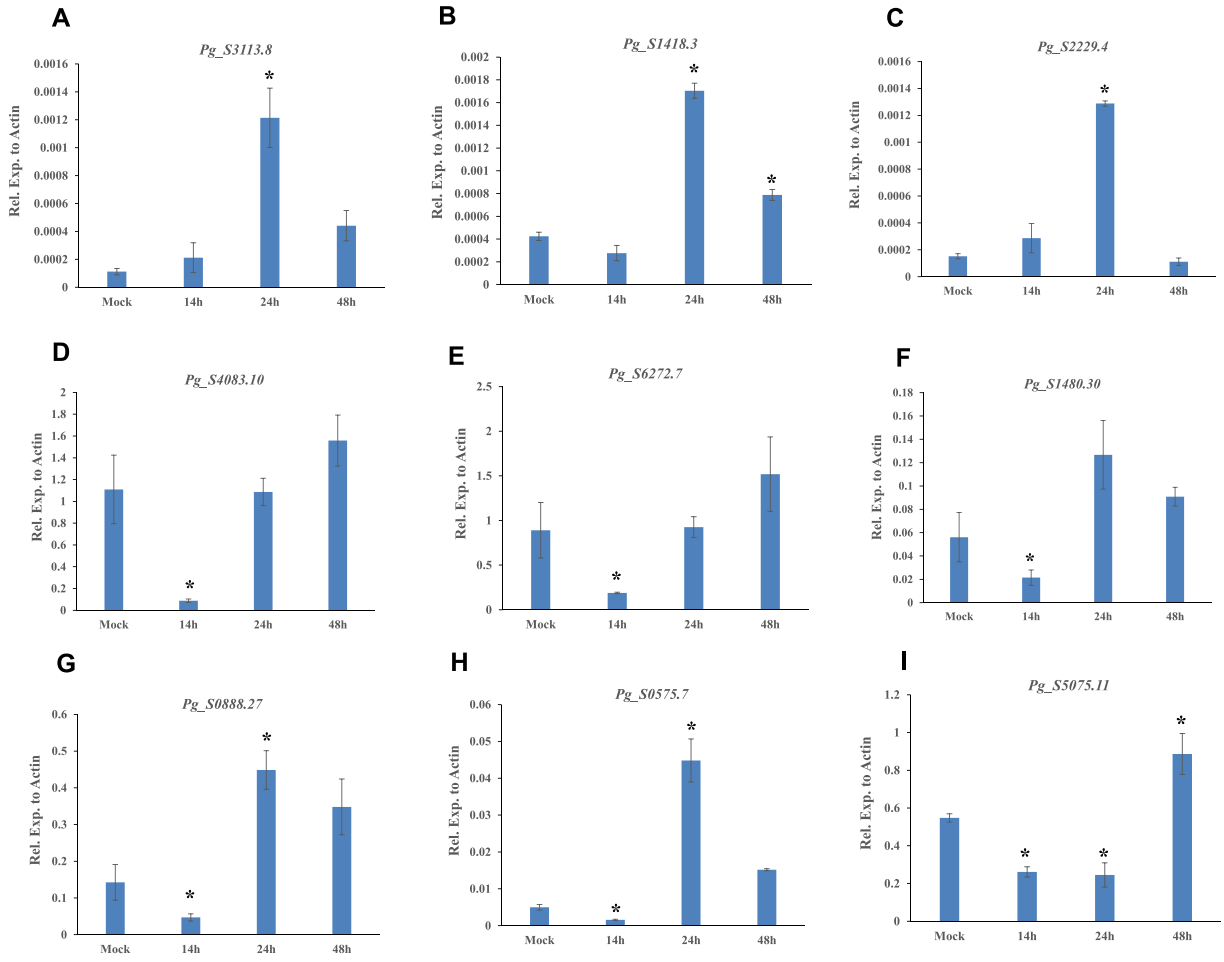

Fig. 2. Expression pattern of AP2/ERF TFs in ginseng response to *B. cinerea* infection at different time. *Pg_S3113.8*: ethylene-responsive transcription factor 4 like; *Pg_S1418.3*: ethylene responsive transcription factor 2a; *Pg_S2229.4*: ethylene-responsive transcription factor 4 like; *Pg_S4083.10*: ethylene responsive transcription factor 2 b; *Pg_S6272.7*: ethylene-responsive transcription factor 4 like; *Pg_S1480.30*: ethylene responsive transcription factor 1 b; *Pg_S0889.72*: ethylene responsive transcription factor 1a; *Pg_S0575.7*: ethylene-responsive transcription factor 2; *Pg_S5075.11*: ethylene responsive transcription factor 1a. The genes were annotated on the Ginseng Genome Database basing on BlastP analysis. All data were normalized to the expression of ginseng *Actin*. Error bars represent SD of three biological replicates. Asterisks indicate significant differences between mock and *B. cinerea* treated (*, $P < 0.05$; two-tailed *t*-test).

The diameters were also significantly smaller in kaempferol, quercetin and luteolin treated fungal growth than CK at 24hpi (Fig. 5B). The inhibition rates were about 46% for quercetin, 25% for kaempferol, and 22% for luteolin at 24 h after incubation, respectively (Fig. 5C). These results showed that kaempferol, quercetin and luteolin could inhibit fungal growth which further indicated certain components in flavonoids have antifungal activity. The hesperetin could not significantly inhibit *B. cinerea* growth at both 24hpi and 48hpi as the colony sizes were as similar as that on PDA (Fig. 5B). At 48 h, the colony size of kaempferol, quercetin and luteolin - treated fungus remained smaller than CK (Fig. 5B). As consequence, the inhibition rates were different (Fig. 5C). Considering the reduction of kaempferol, quercetin and luteolin during *B. cinerea* - ginseng interaction, the fungus very likely developed a strategy to suppress the biosynthesis of these antifungal metabolites.

3.4. *BcQdo gene involved in flavonoids catabolizing and △BcQdo showed reduced disease development*

Since flavonoids play a role in ginseng defense towards *B. cnierea* in vitro and these compounds are decreased at the early fungal infection stage, we next want to know how flavonoids was repressed by *B. cinerea*. The quercetin dioxygenase (Qdo) in *Sclerotina sclerotiorum* was reported to catalyze flavonoids in *A. thaliana* [35]. A *BcQdo* was identified with higher similarity with *SsQdo* (Fig. 6A). We hypothesized *B. cinerea BcQdo* involved in repressing ginseng flavonoids mediated defense.

Gene deletion analysis of *BcQdo* in *B. cinerea* indicated *△BcQdo* caused tiny lesion size in ginseng leaves compared with B05.10 (Fig. 6B, 48 and 60 and 72 hpi; Fig. S9). The lesion sizes in *△BcQdo* infected ginseng leaves were smaller than B05.10 (Fig. 6C). This indicated *BcQdo* involved to *B. cinerea* virulence and *△BcQdo* mutants reduced ginseng disease development. In addition, *△BcQdo* also delayed disease development in soybean at early stage (Fig. 6D, 24 and 36 hpi). The complement lines of *△BcQdo*-C restored wild-type B05.10 virulence both in soybean and ginseng (Fig. 6E; Fig. S10; Fig.S11).

To determine if deletion of *BcQdo* affected flavonoid level, we added quercetin (1.0 mg/ml) to conditional medium (CM) medium and then cultured with *B. cinerea* B05.10 and *△BcQdo* mutants. Medium from *B. cinerea* cultures were collected and analyzed at 0 h–48 h after quercetin added. Higher levels of the quercetin were recovered from the medium colonized by *△BcQdo* mutants

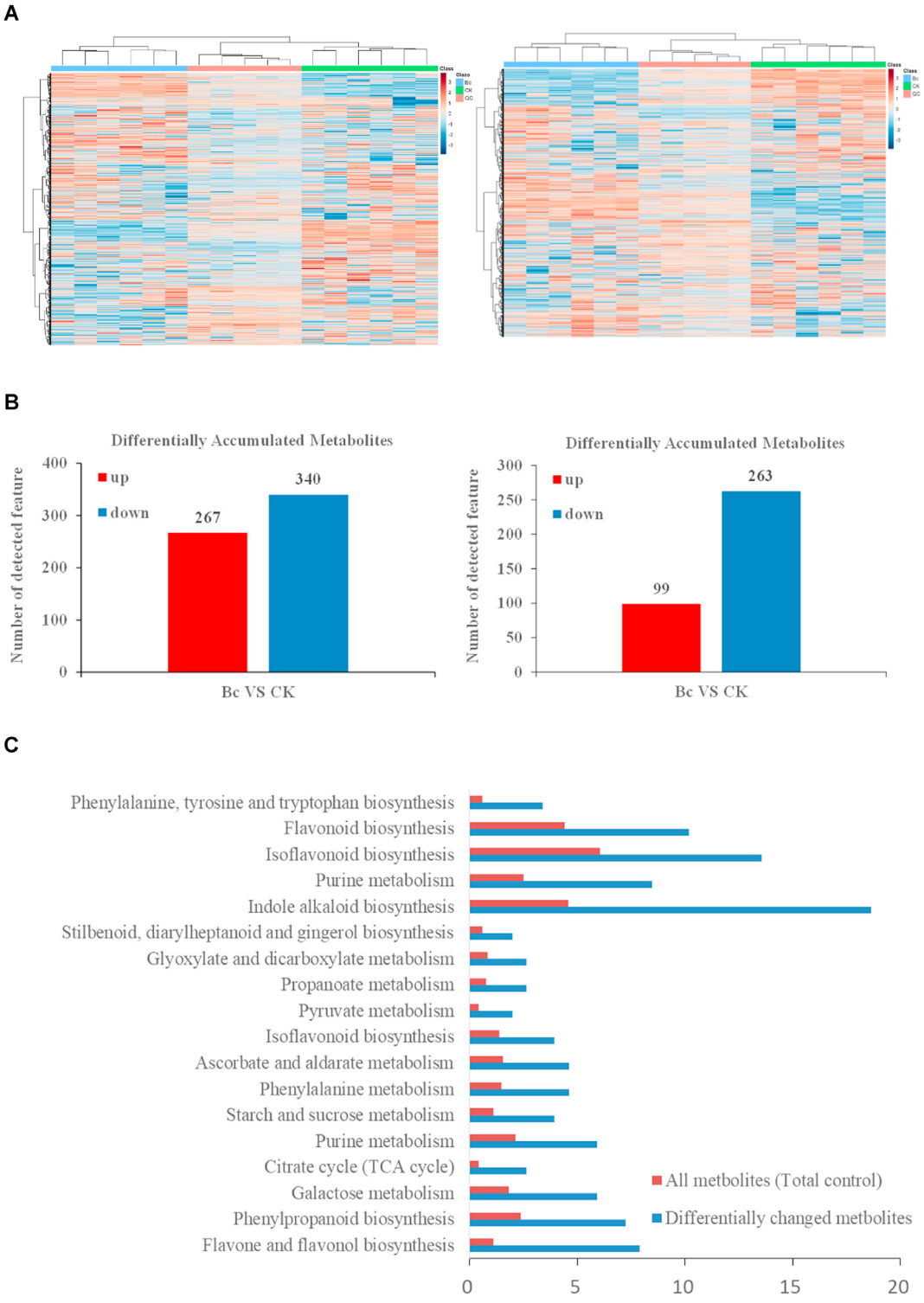

Fig. 3. Untargeted metabolite profiling reveals the variations in the abundance of metabolites in ginseng after *B. cinerea* B05.10 infection. (A) Heatmap of the metabolites identified in the metabolome of mock treatment (CK) or *B. cinerea* B05.10 (*Bc*) treated ginseng (n = 12). The heatmap scale ranges from −3 to +3 on a $\log_2$ scale. Left: POS model; Right: NEG model. (B) Numbers of differentially changed metabolites (≥2-fold; P ≤ 0.05) in ginseng at 14 h after mock treatment (CK) or *B. cinerea* B05.10 (*Bc*) treated. Left: POS model; Right: NEG model. (C) GO analysis of differentially changed metabolites. Numbers indicate the percentages of metabolites belonging to each GO category.

compared to B05.10 at 20 h and 40 h (Fig. 6F). This result indicates the degradation of quercetin was inhibited by the loss of BcQdo.

4. Discussion

B. cinerea caused mold diseases on hundreds of plant species. Little is known about the molecular mechanisms controlling the interaction between medicinal plants such as *P. ginseng* defense

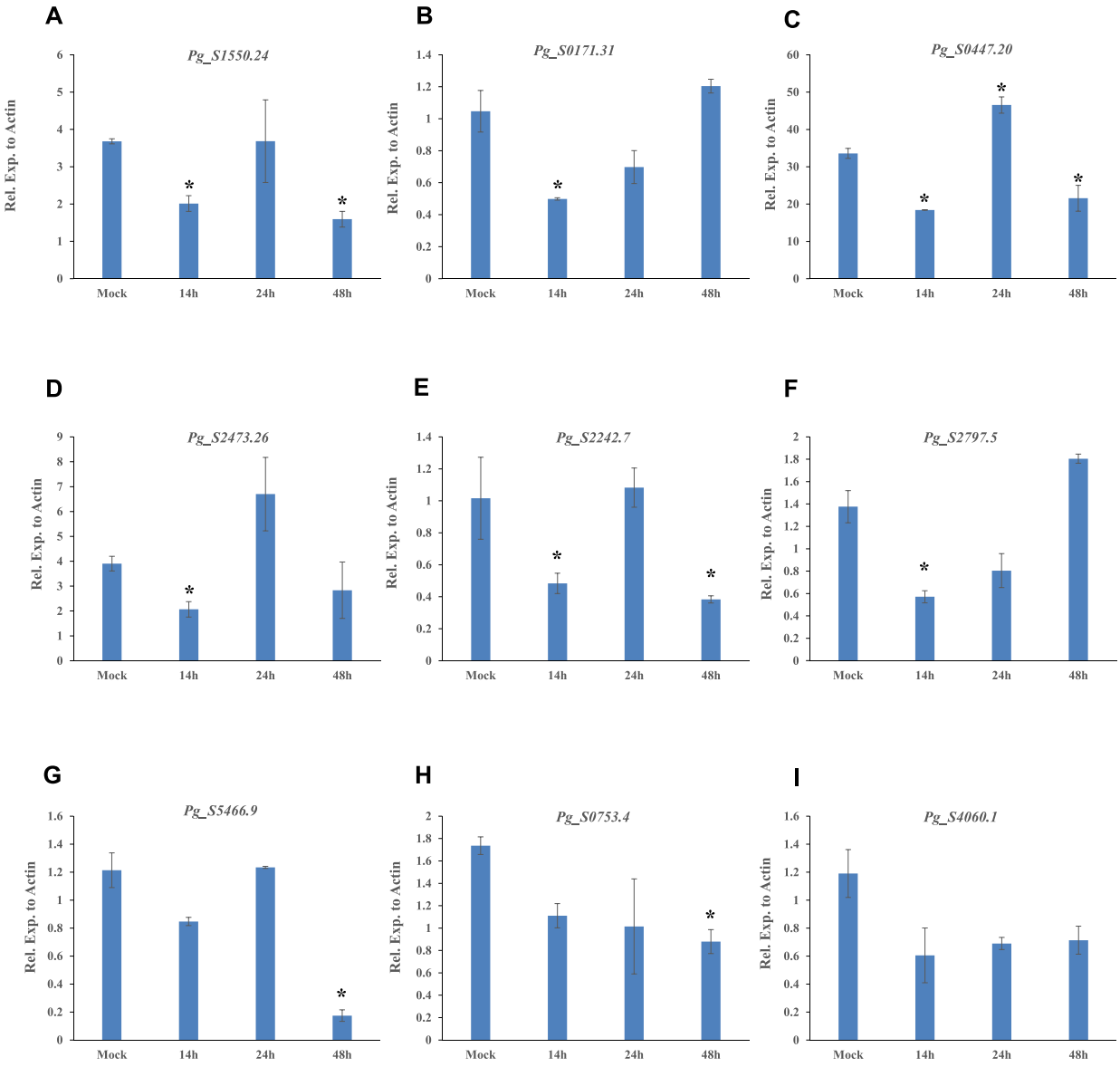

Fig. 4. Expression of genes involved in regulation of flavonoid biosynthetic process. *Pg_S1550.24*: hydroxycinnamoyl transferase; *Pg_S0171.31*: hydroxycinnamoyl CoA quinate transferase 2 like; *Pg_S0447.20*: isoflavone reductase-like; *Pg_S2473.26*: phenylcoumaran benzylic ether reductase; *Pg_S2242.7*: leucoanthocyanidin dioxygenase-like; *Pg_S2797.5*: naringenin,2-oxoglutarate 3-dioxygenase; *Pg_S5466.9*: phenylcoumaran benzylic ether reductase; *Pg_S0753.4*: flavanone 3-hydroxylase-like protein; *Pg_S4060.1*: cinnamate 4-hydroxylase. The genes were annotated on the Ginseng Genome Database basing on BlastP analysis. All data were normalized to the expression of ginseng *Actin*. Error bars represent SD of three biological replicates. Asterisks indicate significant differences between mock and *B. cinerea* treated (*, $P < 0.05$; two-tailed *t*-test).

towards *B. cinerea*. Pathogen invasion usually induces a profound and dynamic transcription reprogramming of plant gene expression. The universal defense response employed by plants involves, activation of complex phytohormone signaling networks, TFs and phytoalexins [36]. For example, the TFs are reported to regulate secondary metabolism such as flavonoids [37–40]. Transcriptional regulation of the flavonoid pathway via MYBs are widespread among plants [37,38]. In *Populus*, MYB115 and MYB134 regulate proanthocyanidin synthesis [40]. In *A. thaliana*, several MYBs are regulators of flavonoid biosynthesis while some MYBs regulate genes in glucosinolates biosynthesis [39,41]. GlMYB4 and GlMYB88 could positively regulate the synthesis of flavonoids in licorice cells [42]. In addition, MYCs, WRKYs and ERFs also involved in plant secondary metabolites [39,40]. Except TFs, phytohormones like JAs are signals in the biosynthesis of different groups of secondary compounds including anthocyanins, glucosinolates and artemisinin [39,40]. JAs are involved in the regulation of JA-dependent TFs such as MYBs and MYCs, which activate or repress the expression of essential genes in the biosynthesis of secondary compounds [40]. In *Ginkgo biloba*, MeJA induced the accumulation of flavonoids such as quercetin, quercetin-4-glucoside and luteolin [43]. In *Glycyrrhiza uralensis*, MeJA significantly induced the expression of *GlMYBs* [42]. The JAs-responsive TFs mediating secondary metabolite biosynthesis are associated with plant defense. However, the virulent pathogens have developed capabilities to manipulate or subvert plant defense for their own benefits [19,44].

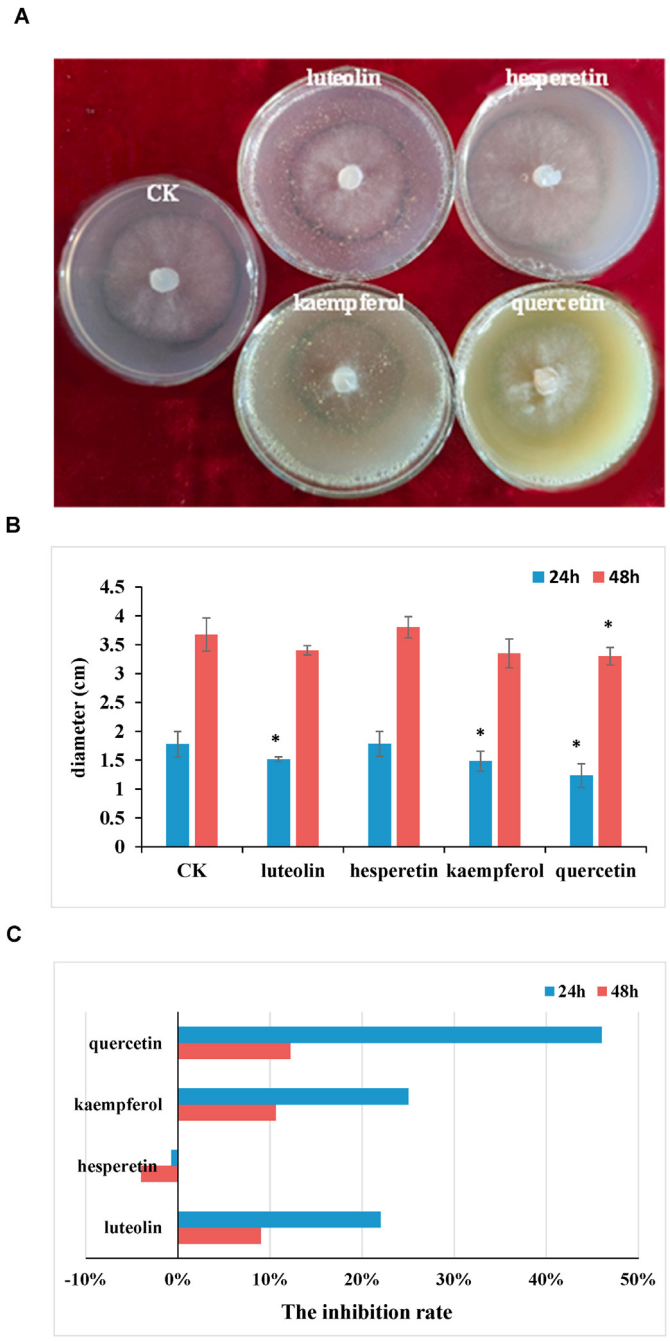

Fig. 5. Antifungal activity of differentially changed metabolites in flavonoids pathway. (A) Mycelial plugs of the B05.10 was inoculated on PDA medium with different compounds including kaempferol, quercetin, hesperetin and luteolin. The growth of the fungi was observed and photographed at 48 h. (B) The colony diameter was determined at 24 h and 48 h, respectively. Asterisks indicate significant differences between untreated (CK) and flavonoids treated (*, $P < 0.05$; two-tailed *t*-test). (C) The relative inhibition rates of different flavonoids towards B05.10 were determined at 24 h and 48 h, respectively. All data represent means ± SD from at least three independent experiments.

4.1. B. cinerea promotes disease development in ginseng by suppression of many defense related genes expression at the early infection stage

Since plant defense compounds are regulated by TFs, a potential way for pathogens to disturb defense is to target TFs [19]. In this work, during *P. ginseng* - *B. cinerea* early interaction, transcriptions of many TFs were decreased (68%, 86 out of 126), suggesting this fungus might target and modulate the expression of TFs. By this, the fungus manipulates ginseng defense responses thereby benefits its infection. Similarly, in *Medicaga truincatula*, ERF19 is targeted by a secreted protein from the arbuscular mycorrhizal fungus *Glomus intraradices* [45]. The *Xanthomonas* effector XopD interacts with ERF4 in tomato and MYB30 in *Arabidopsis*, respectively. MYB30 is a key regulator of multiple hormones signaling pathways [46]. The PAMP flg22 induces alternatively polyadenylated forms of ERF4 transcript in *Arabidopsis*, thereby influenced its defense related functions [47]. In addition, some TFs in ginseng were also up-regulated by *B. cinerea*, which might active downstream compounds and play a role in defense.

In addition, many genes (72%, 21 out of 29) involved in JA/ET signaling were suppressed by *B. cinerea* during infection ginseng. Metabolite analysis confirmed the reduction of JA compounds. Therefore, *B. cinerea* has ability to suppress the JA/ET pathway in ginseng. Similar reports have shown in other pathogen - host interactions. For instance, *S. sclerotiorum* produced a secretory protein SSITL that suppressed JA-dependent defenses [48]. A second secretory protein SsCP1 in *S. sclerotiorum* could directly targeted plant PR1, the transgenic plant expressed *SsCP1* had increased SA levels [49]. A secreted protein MiSSP7 from Laccaria bicolor prevented JA dependent degradation of PtJAZ6, resulting in the repression of JA induced genes [50]. In this work, the transcriptome analysis highlighted *B. cinerea* suppressing ginseng defense responses, thereby lower accumulation of antifungal compounds such as flavonoids. Interestingly, transcriptome analysis of *Arabidopsis* during *B. cinerea* infection also indicated certain transcriptional responses such as TGA3 with a positive role in defense were downregulated during infection [51].

The necrotroph *B. cinerea* has an extremely broad host range including *P. ginseng* and *P. quinquefolis*, two of the important medicinal plants [5]. To predict gene functions and to identify novel components during host - *B. cinerea* interaction, multiple data types from multiple species are required [5]. RNA-seq technology has been used to profile *B. cinerea* gene expression during interaction with different hosts and shown that gene expression is highly correlated between the different infections, indicating a common infection strategy or function of the pathogen [52]. In addition, the diverse expression of *B. cinerea* genes during interaction with different hosts indicated this fungus may selectively attack plants depending on the host tissue [52]. We observed 60% of ginseng SSTF genes were repressed by *B. cinerea* at the early interaction stage, which is different with other reports, suggesting a different infection strategy or function of this pathogen towards ginseng. Thereby, the transcriptome profiling of *P. ginseng* during *B. cinerea* infection will not only help to uncover the commonalities in plant defense responses, but also help highlight the diversity of fungal infection strategy during interaction with medicinal plants. Further studies will identify which factors in *B. cinerea* are involved in suppressing ginseng defense responses such as TFs, JA/ET and so on.

4.2. Flavone plays a role in P. ginseng defense towards B. cinerea

Previous works indicated that plants contained high concentrations of antifungal compounds, some of which provided the plant with a basic resistance against fungal pathogens [53,54]. The changes in flavonoids have been isolated from various species to date [34,55,56]. Here, metabolites analysis of ginseng leaves indicates many of secondary metabolites in flavone and flavonol biosynthesis pathway, isoflavonoid biosynthesis are affected by *B. cinerea*. The single compound of flavones could inhibit *B. cinerea* growth at early stage. As a similar, quercetin 3-*O*-glucoside had been reported to exhibit cytotoxic and antimicrobial activity

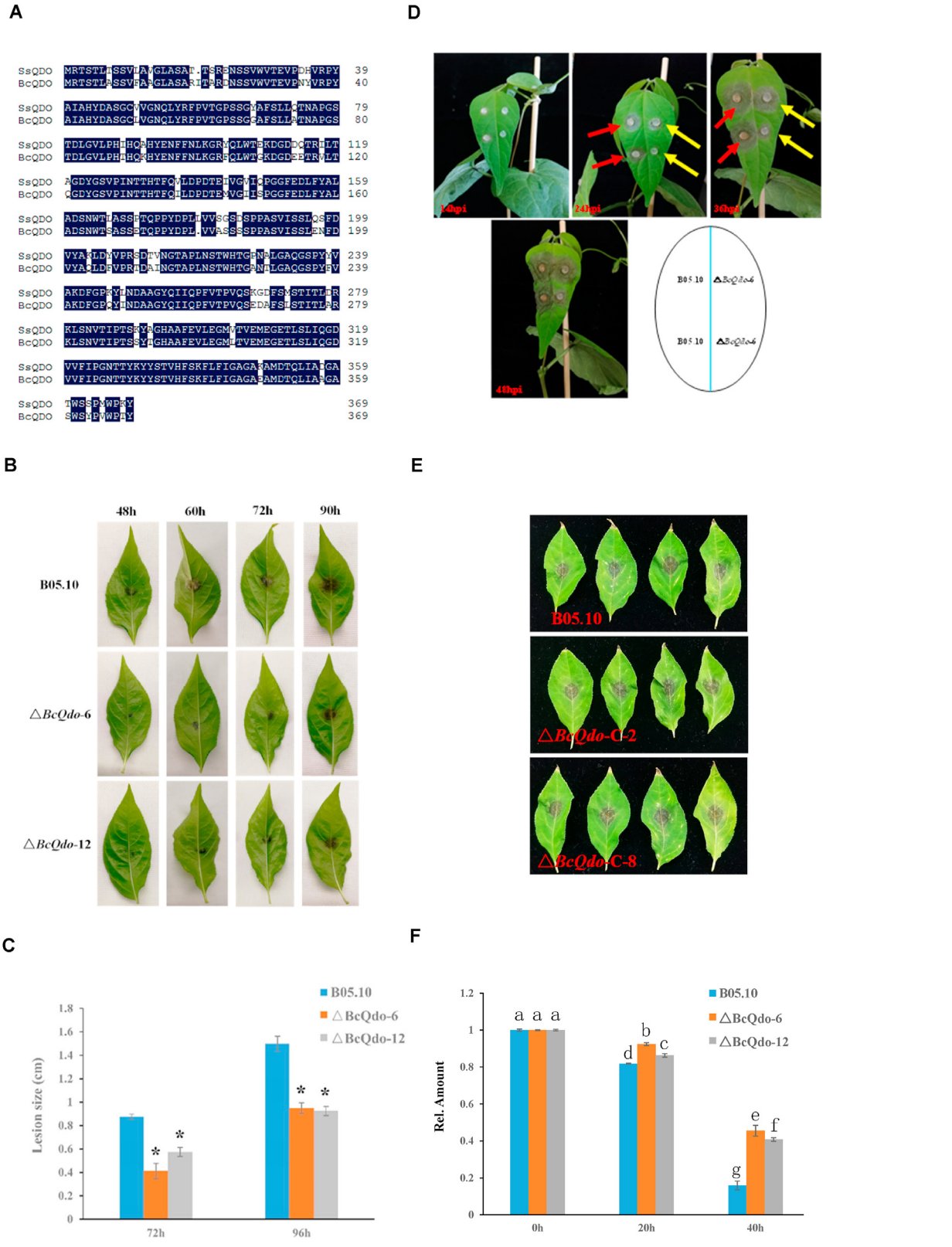

Fig. 6. *BcQdo* mediated *B. cinerea* virulence. (A) Alignment of BcQdo and SsQdo. Alignment of protein sequences was done with Clustal W. (B) Virulence of Δ*BcQdo* mutants (Δ*BcQdo-6*, Δ*BcQdo-12*) in ginseng leaves. The indicated strains were incubated on ginseng leaves for 48 h, 60 h, 72 h and 90 h, respectively. (C) Infection biomass analysis of *B. cinerea* indicated strains on ginseng leaves. The lesion diameters were measured at 72 and 96 h after infection, respectively. (*, $P < 0.05$; two-tailed *t*-test). (D) Virulence of Δ*BcQdo* mutants (Δ*BcQdo-6*, Δ*BcQdo-12*) in bean leaves. The mutant and B05.10 were incubated on bean leaves for 14 h, 24 h, 36 h and 48 h, respectively. The arrows showed different lesion sizes infected by B05.10 (red) and Δ*BcQdo* mutant (yellow) at 24 hpi and 36 hpi, respectively. (E) Phenotypes of Δ*BcQdo*-C (Δ*BcQdo*-C-2, Δ*BcQdo*-C-8) showed recovered pathogenicity in ginseng. (F) *B. cinerea* BcQdo involved in quercetin degradation. Compared to *B. cinerea* B05.10, deletion of *BcQdo* gene results in lower quercetin degradation in the CM medium with Δ*BcQdo* mutants (Δ*BcQdo-6*, Δ*BcQdo-12*). The CM medium with 1.0 mg/ml quercetin was used as control (0 h), the relative amount of quercetin was measured after B05.10 and Δ*BcQdo* mutants (Δ*BcQdo-6*, Δ*BcQdo-12*) incubation at different time, respectively. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA followed by Tukey's post-hoc test).

[57,58]. The quercetin and cyanidin aglycones were shown to inhibit *Colletotrichum gloeosporioides* hyphal growth and conidial germination [59]. The red mango fruit contained more anthocyanin and flavonoids than the green mango enhanced the antifungal activity against *C. gloeosporioides* [56]. Our work reveals kaempferol, quercetin and luteolin, but not hesperetin, play a role in ginseng defense to *B. cinerea*.

Since some flavonoids are reported to have antifungal activities, the pathogen might target the compounds. Here, a *BcQdo* was involved in *B. cinerea* - ginseng interaction by degradation of flavone. △*BcQdo* mutants reduced its ability to degrade quercetin and were less virulent to ginseng and other plant. Other findings have also demonstrated that pathogens can target plant secondary metabolite. For example, *S. sclerotiorum* has developed a way to circumvents flavonoid defense by catabolizing flavonol glycosides and aglycones [35]. *B. cinerea* MFS transporter mfsG involved in detoxify isothiocyanates and was required for pathogenicity. The *mfsG* mutant was deficient in efflux isothiocyanates and was less virulent to glucosinolates-containing plants [60]. Here, the *B. cinerea BcQdo* targets and suppresses flavonoids - mediated defense to promote disease development in ginseng would help us to understand the regulatory mechanism between this medicinal plant and the fungal pathogen.

5. Conclusions

In a conclusion, our studies revealed that the virulent *B. cinerea* strain B05.10 represses ginseng defense genes expression and unbalance antifungal flavone biosynthesis thereby leading to clearly reduced early immune responses. Further results indicated *BcQdo* contributes to pathogenicity as the virulence was partly reduced and the antifungal flavone degradation was affected by △*BcQdo* mutant.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2022.03.005.

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