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Research Article

Botrytis cinerea hypovirulent strain △*BcSpd1* induced *Panax ginseng* defense

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ABSTRACT

Background: Gray mold, caused by *Botrytis cinerea*, is one of the major fungal diseases in agriculture. Biological methods are preferred over chemical fungicides to control gray mold since they are less toxic to the environment and could induce the resistance to pathogens in plants. In this work, we try to understand if ginseng defense to *B. cinerea* could be induced by fungal hypovirulent strain △*BcSpd1*. *BcSpd1* encodes $Zn(II)_2Cys_6$ transcription factor which regulates fungal pathogenicity and we recently reported △*BcSpd1* mutants reduced fungal virulence.
Methods: We performed transcriptomic analysis of the host to investigate the induced defense response of ginseng treated by *B. cinerea* △*BcSpd1*. The metabolites in ginseng flavonoids pathway were determined by UPLC-ESI-MS/MS and the antifungal activates were then performed.
Results: We found that △*BcSpd1* enhanced the ginseng defense response when applied to healthy ginseng leaves and further changed the metabolism of flavonoids. Compared with untreated plants, the application of △*BcSpd1* on ginseng leaves significantly increased the accumulation of p-coumaric acid and myricetin, which could inhibit the fungal growth.
Conclusion: *B. cinerea* △*BcSpd1* could effectively induce the medicinal plant defense and is referred to as the biological control agent in ginseng disease management.

1. Introduction

Plant and microbe/pathogen co-evolve in nature. Plant defense to pathogen has a powerful immune system consisting of two interconnected tiers [1,2]. One tier recognizes microbe/pathogen associated molecular patterns (MAMPs/PAMPs), by the pattern recognition transmembrane receptor (PRRs) and is known as MAMP/PAMP-triggered immunity (MTI/PTI), leading to a basal level of the immune response. Other tier responds to pathogen derived specific effectors, which can be recognized by NB-LRR family proteins and is called effector-triggered immunity (ETI) [1–4]. ETI is also named the R-gene-dependent resistance or gene-for-gene mediated resistance [1]. In addition, plant defense responses are also controlled by induced disease resistance [5,6]. Plants possess various inducible defense mechanisms for protection against potential pathogens [7–10]. For example, systemic acquired resistance (SAR) is activated by a wide range of pathogens [11], while induce systemic resistance (ISR) in plant is activated by certain nonpathogenic rhizobacteria or fungi during colonization of plant roots [12]. Both SAR and ISR are effective against different types of pathogens, and are typically characterized by a restriction of microbe growth and a suppression of disease development compared with non-induced plants [7].

In all cases, the resistance of the plant is associated with transcriptional reprogramming, which is closely related to phytohormones, secondary metabolites and transcription factors [13–15]. Whereas SAR requires endogenous biosynthesis of salicylic acid (SA), the signaling in ISR is independently of SA and requires intact responsiveness to jasmonic acid (JA) and ethylene (ET) [7,12,16]. The role of hormones in plant-pathogen interaction is different depending on the invaders and plant species. For instance, whereas SA has been traditionally associated with defense against the biotrophic or hemibiotrophic pathogens, the JA/ET signaling appears to

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be more important to the necrotrophic pathogens or insects [13]. Further researches indicated JA signaling was also involved in the plant-biotrophic interaction while SA/JA pathways collaborated together during ETI [17,18]. Additionally, it has been demonstrated that quickly accumulation of phytoalexins is integral to plant defense [7,19]. The structures of phytoalexins vary among different plant families and include flavonoids, terpenoids and indoles [20]. Next to the plant hormones and phytoalexins, transcription factors (TFs) play important roles in plant defense. Many works indicated transcriptional re-programming of plant cells mediated by TFs is a crucial step to mount an efficient defense response [14].

Botrytis cinerea is a broad host-range necrotrophic fungal pathogen that can affect more than 1,400 plant species [21]. Towards to the fungal pathogenic factors, strategy associated with biological management is thought to be a safe alternative and highly practicable for disease management [5,22]. The biological control method uses living organisms to suppress the pest population density to a less abundant or less damaging level [5]. The living organisms that inhibit the pathogen is referred to as the biological control agent that can directly or indirectly cope with the pathogen. The indirectly suppression through the plant-microbe interaction induces SAR and ISR [6,23]. For example, *Trichoderma* species, arbuscular mycorrhizal fungi, ectomycorrhizal fungi, endophytes, avirulent and hypovirulent strains of certain pathogens are beneficial organisms with biocontrol capacity [24–26]. However, the underlying molecular mechanism and biocontrol strategy associated with pathogenicity deficient factors towards broad host-range necrotrophy have not been well analyzed. *B. cinerea* $Zn(II)_2$-Cys_6 transcription factor *BcSpd1* was reported to involve in the fungal infection cushion formation, sclerotium development, biosynthesis of melanin, change of environmental pH and fungal pathogenicity [27]. △*BcSpd1* mutants were less virulent towards Arabidopsis, bean and tomato [27]. Thus, we proposed *B. cinerea* hypovirulent strain △*BcSpd1* may be utilized as a biocontrol agent in the medicinal plant *P. ginseng* which has higher economic values and now suffers from heavy gray mold disease [15,27].

In this work, we examined the induced plant resistance mediated by fungal pathogenic gene mutant △*BcSpd1* from the necrotrophy *B. cinerea*. We performed transcriptomes analysis in the medicinal plant *P. ginseng* leaves upon *B. cinerea* △*BcSpd1* treatment. We compared the data from the same treatments to obtain an integrated understanding of activation of ginseng resistance by induction of defense related genes expression and increased accumulation of antifungal flavonoids.

2. Materials and methods

2.1. Plants and fungi materials

Two-years old ginseng roots were grown in fresh-healthy soil under a microbe free climate chamber (8h/16h, light/dark). One month later, the heath ginseng leaves were used for the infection experiments. *B. cinerea* wild-type strain B05.10 and the *BcSpd1* gene mutant strain △*BcSpd1* were incubated in PDA plate to generate the fresh conidia spores [28,29].

2.2. Fungal incubation and plant samples collection

For infection phenotype, ginseng leaves were incubated with two droplets of $2.5*10^5$ mL^{-1} *B. cinerea* indicated spores (B05.10 and △*BcSpd1*) for 3 days. For RNA sequencing, the ginseng leaves were infected with *B. cinerea* indicated spores by spraying method. Ginseng leaves without fungal spores were used as control (CK). All leaves were harvested at indicated times with 3 replicates. The samples collected with same treatments were harvested for flavonoid detection (4 replicates). For qPCR assay, plants were infected with *B. cinerea* indicated strains and collected at different time points. All the samples were frozen immediately with liquid N_2 and kept at −80 °C.

2.3. Library construction for RNA sequencing

Total RNA isolation, purification and monitoring, and cDNA library construction and sequencing were performed as previously [27,28]. A total of nine samples were sequenced.

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

As indicated in our previous work, clean reads were first obtained by removing the lower quality reads from the raw data [15,27]. Reference genome was got from the website (http://ginsengdb.snu.ac.kr/data.php). Index of the reference genome was built and paired-end clean reads were aligned to the reference, and the FPKM of each gene was finally calculated based on the length and reads count mapped to the gene as indicated [15].

2.5. Identification of differentially expressed genes (DEGs) and GO/KEGG enrichment analysis

Differential expression analysis of all samples was performed [27,28]. The differentially expressed genes (DEGs) were selected with log2 (fold change) > 1 or log2 (fold change) <-1 and with statistical significance (p value < 0.05) by R package. GO enrichment analysis of DEGs was implemented by the GOseq R package, while KEGG is used for understanding high-level information, basing on large scale molecular datasets [15,27].

2.6. Real-time quantitative PCR

Real-Time quantitative PCR were performed as previously indicated [15,27]. The *PgActin* was used for normalization. All analyses were repeated three times using biological replicates. Primer sequences are listed in Table S1.

2.7. Chemical analysis

Frozen ginseng leaves were grounded in liquid nitrogen with a mortar and pestle. 0.1 g of the sample was extracted with 0.3 mL methanol: acetonitrile: water (2:2:1, v/v) in an ultrasonic sonicator for 30 min at 4 °C for twice. The sample was incubated at −20 °C for 1 hour to depose the protein. After centrifugation (14,000 rpm, 20 min, 4 °C), the supernatants were collected, freeze drying and stored at −80 °C. Then the supernatants were filtered through a 0.22 μm organic membrane and collected for HPLC analysis.

The chemicals such as flavonoids were separated on a Waters BEH C18 column (2.1 mm × 150 mm, 1.7 μm) at a flow rate of 1.0 mL min^{-1}. The separation used solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The auto-sampler temperature was set to 4 °C and 2 μL of the extraction was injected for analysis. Mass spectrometric detection was performed using an ABSCIEX QTRAP 6500 mass spectrometer coupled with an electrospray ionization (ESI) source. The multiple reaction monitoring scan mode was used to quantify the individual flavonoid compounds.

2.8. Ginseng leaves extracts isolation and the antifungal activity analysis

The leaves were harvested from two years old ginseng. The samples were thoroughly dried at 50 °C and ground into powder. The powder was then passed through a filter (0.42 mm) and 0.5 g of the fine powder was precisely weighed, and mixed with 25 mL of 100 % methanol. The mixture was ultrasonicated at 150 W and 40 kHz for 30 min. After centrifugation at 5000 rpm for 5 min, the supernatant was collected and concentrated for antifungal activity analysis.

The effects of the flavonoids or the ginseng leaf extracts on mycelia growth in *B. cinerea* B05.10 was assessed using the mycelial plugs growth rate method in PDA. The compounds were dissolved in methanol and then 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 [15].

2.9. Statistical analysis

The analysis of variance was used Statistical Product and Service Solutions (SPSS) 18 software (IBM). The differences were considered significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, respectively. All the data are represented as the mean ± SEM of at least three replicates.

3. Results

3.1. B. cinerea △BcSpd1 is hypovirulent towards P. ginseng

B. cinerea B05.10 is virulent towards *P. ginseng* by repressing host defense-related genes at early stage as reported recently [15]. Further work indicated the *B. cinerea* △*BcSpd1* mutants reduced fungal virulence towards *Arabidopsis thaliana*, tomato and bean [27]. Here we first tested its virulence towards ginseng. The wild-type B05.10, two mutants of △*BcSpd1*, the gene complement line were used. Three days post infection, the typical necrosis appeared in both B05.10 and the complement line incubated ginseng leaves while the lesions were tiny in △*BcSpd1* mutants treated plants (Fig. 1A). These indicated △*BcSpd1* is hypovirulent towards *P. ginseng*, which is consistent with our previous reports [27].

3.2. B. cinerea △BcSpd1 induced P. ginseng defense response

To see if *B. cinerea* △*BcSpd1* involved in activating ginseng defense response and contributed to plant immunity, the antifungal activities were detected upon △*BcSpd1* treatment. Ginseng leaves were treated with *B. cinerea* B05.10 and △*BcSpd1* for 14 hours, 24 hours and 48 hours, respectively. The leaves without fungal spores were used as control (mock treated). Next, ginseng crude extractions (GCE) from the control plants, B05.10 spores spray-infected plants (B05.10-treated, 14, 24 and 48 hpi), and △*BcSpd1* spores spray-infected plants (△*BcSpd1*-treated, 14, 24 and 48 hpi) were collected, extracted, and their anti-fungal activities were analyzed. As shown in Fig. 1B and C, all the GCEs (1mg ml^{-1}) inhibited the growth of B05.10 after growing for 48 hours compared with CK (PDA plate without GCE). As expected, the inhibition rate was higher by △*BcSpd1*- treated GCE (PDA plate with GCE-14pi) at the early stage compared with B05.10- treated (Fig. 1D, the red arrow labeled). Then, the inhibition rate by GCEs extracted at the late stages (24 and 48 hpi) were lower in △*BcSpd1* than B05.10 (Fig. 1D, the green arrow labeled). These data indicated △*BcSpd1*- treated ginseng has stronger antifungal activity at early stage (14 hpi).

Next, to see if *B. cinerea* △*BcSpd1* induced plant resistance, *P. ginseng* were grown in the pots and pre-treated with spores of B05.10 and △*BcSpd1*, respectively. 14 hours after pre-treatment, intact plants were incubated with mycelial plugs of *B. cinerea* B05.10. As indicated in Fig. S1, △*BcSpd1* pre-treated plants strongly enhanced the resistance towards B05.10 (Fig. S1C, F) compared with CK (Fig. S1A, D) and B05.10 pre-treated plants (Fig. S1B, E). It indicated △*BcSpd1* induced ginseng defense responses, and the response perhaps due to the differentially changed genes and metabolites triggered by △*BcSpd1*.

3.3. Transcription analysis of P. ginseng differentially expressed gene upon B. cinerea △BcSpd1 infection

To understand the molecular mechanism of *B. cinerea* △*BcSpd1* in inducing ginseng immunity, RNA-sequencing was performed. Ginseng leaves incubated with *B. cinerea* △*BcSpd1* were harvested at 14 hours (△*BcSpd1* 14h) and 24 hours (△*BcSpd1* 24h), respectively. For the control, ginseng leaves without any treatment were harvested. RNA-seq data were then analyzed. A total of 382 million validated high-quality reads were obtained from all nine libraries (Table S2). The reads were aligned to the *P. ginseng* genome. The raw sequence data have been submitted to the NCBI Short Read Archive with accession number GSE221931.

To identify genes involved in ginseng response to *B. cinerea* △*BcSpd1* at the genome-wide level under different time, we compared differentially changed genes (statistically significantly, $P \leq 0.05$, altered at least two-folds, SSTF) between △*BcSpd1*-treated (△*BcSpd1*) and un-treated (CK sample) ginseng. At 14 hour, a total of 1619 SSTF genes were identified in △*BcSpd1*- treated plants compared with CK, with 998 of them are increased while 621 of them are decreased (Fig. 2A). Interestingly, a total of 7743 SSTF genes were identified at 24 hour compared with CK. This indicates more genes were affected in responding to △*BcSpd1* treatment from time to time. About 4229 genes were induced while 3514 genes were repressed, suggesting more genes were induced (Fig. 2A). The comparison of transcripts between 24 hour and 14 hour was also performed. As indicated in Figs. 2A and 9761 SSTF genes were observed with 5286 of them was increased while 4475 of them was decreased, further confirmed the induction of host genes by △*BcSpd1* at 24 hour.

Next, when compared the SSTF genes both at 14 hour and 24 hour, 660 genes were observed (Fig. 2B). It indicated 660 genes were consistently significantly affected upon △*BcSpd1* treatment, not only at 14 hour but also at 24 hour. Heatmap analysis was performed and different expression patterns among these 660 genes were shown in Fig. 2C. Several groups of genes were clustered together. The genes in group I was increased in ginseng upon △*BcSpd1* infection at both 14 hour and 24 hour as the lowest expression level was in CK. For the genes in group II, the highest expression level was observed at 14 hour while the lowest level was at 24 hour, suggesting the early role of these genes. The genes in group III were gradually decreased from CK to 24 hour while the genes in group V were decreased at both 14 hour and 24 hour. As a difference, the genes in group IV were decreased at 14 hour while increased to an even higher level at 24 hour. These data indicated △*BcSpd1* infected altered genes expression in ginseng which might contribute to plant defense.

To confirm the induction of ginseng defense response by△*BcSpd1*, the gene expression pattern was additionally analyzed in B05.10 and △*BcSpd1* mutants infected *P. ginseng* at different time points by qPCR, respectively. As shown in Fig. 3, expression level of several genes encoding TFs such as MYBs, ERFs etc were increased in △*BcSpd1* infected ginseng at 14 hour compared with B0510 infected plants. Similarly, expression of genes in JA/ET signaling and

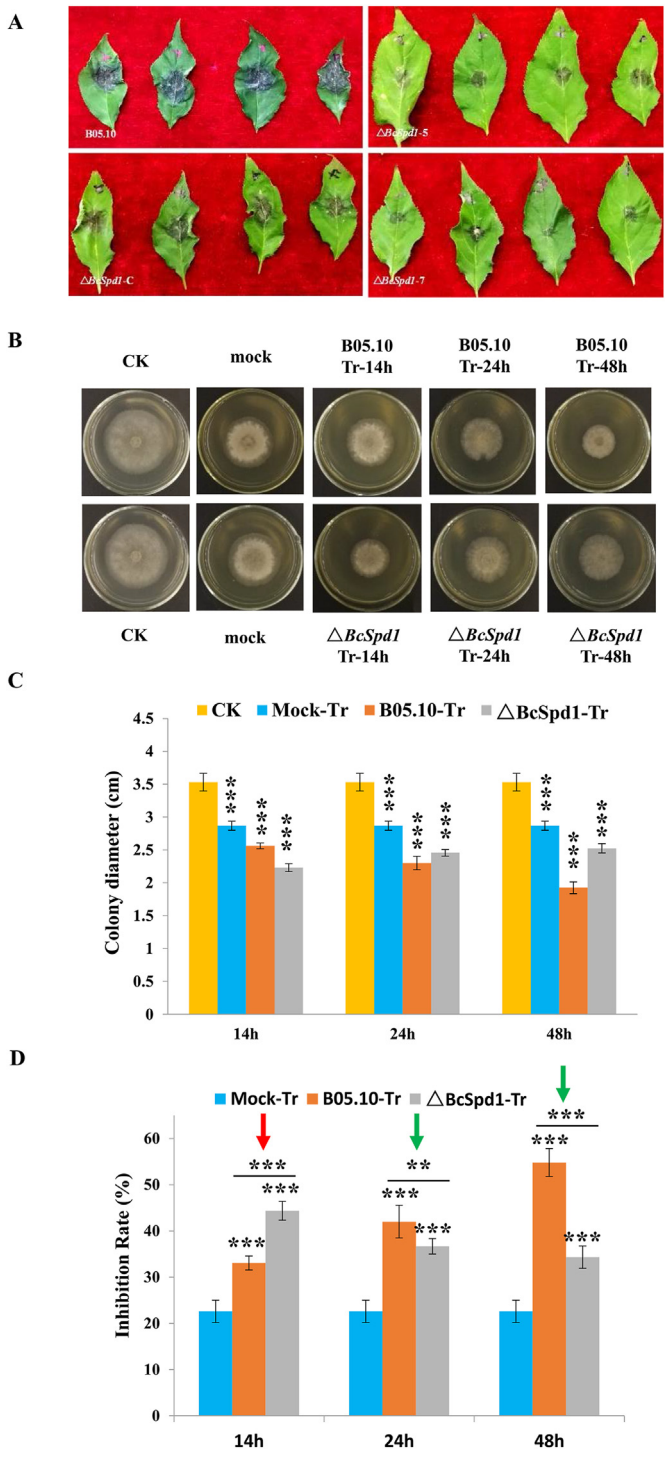

Fig. 1. *Panax ginseng* defense response changed upon *B. cinerea* B05.10, △*BcSpd1* and △*BcSpd1*-C infection. (A) *P. ginseng* susceptibility altered by △*BcSpd1* treatment. Photographs were taken 3 days post-infection. Wild-type strain B05.10 presents large lesions compared with △*BcSpd1-5* and △*BcSpd1-7* infected leaves that show small lesions or no necrotic symptoms on leaves. The complement line △*BcSpd1*-C recovered from wild-type pathogenicity. (B-D) Antifungal analysis of different ginseng leaf extractions towards *B. cinerea* B05.10 growth. The leaf extractions including mock-treated, B05.10-treated ginseng at different times (14h, 24h and 48hours), Δ*BcSpd1*-treated ginseng at different times (14h, 24h and 48hours). (B) Growth of *B. cinerea* B05.10 on PDA plates with different ginseng leaf extractions (1.0mg mL^{-1}) for 24 hours. PDA plates without any leaf compounds were used as control (CK). (C) Colony diameters of *B. cinerea* B05.10 growth on PDA with different ginseng leaf extractions. Error bars represent SD of at least three biological replicates. Asterisks indicate significant differences between CK and different treatments. (D) Inhibition rates of *B. cinerea* B05.10 growth on PDA upon different treatment compared with CK. Error bars represent SD of three biological replicates. Asterisks indicate significant differences between different treatments (**, P < 0.01; **P < 0.001, two-tailed t-test).

flavone biosynthesis pathway were also partly increased in △*BcSpd1* infected ginseng at early stage compared with the virulent strain B05.10 (Fig. 3). These results indicated that △*BcSpd1*-induced ginseng resistance towards B05.10 perhaps by activating of early defense response genes.

3.4. Flavonoid biosynthesis related genes were enriched in △*BcSpd1*- treated ginseng at 14 hour post infection

Since *B. cinerea* treatment often up- or down- regulation of host genes expressions during interaction, we next analyzed the transcription diversity in ginseng upon *B. cinerea* △*BcSpd1* infection at 14 hour and 24 hour. The differentially expressed genes were analyzed by GO and KEGG methods, respectively.

For the SSTF genes at 14 hour, GO terms about unsaturated fatty acid biosynthetic process, cell wall, NADPH activity and membrane are enriched (Fig. S2A). KEGG analysis indicated the genes associated withbiosynthesis and metabolism are enriched (Fig. S2B). Interestingly, flavonoid biosynthesis took the first grade when analyzing the top20 of KEGG enrichment at 14 hour (Fig. 4A). The enrichment of genes in flavonoid biosynthesis pathway suggests a key role of flavonoid in plant early defense response.

Heatmap analysis was then performed and different expression patterns among 24 genes in flavonoid biosynthesis were shown in Fig. 4B and Dataset S1. Interestingly, two groups of expression patterns were observed. The genes framed in green color indicated the lowest expression level at 14 hour while the genes framed in black color was higher than that in CK (Fig. 4B). The differentially expression of genes at 14 hour suggest a complex role of flavonoid in ginseng defense.

3.5. *P. ginseng* defense related genes were activated upon △*BcSpd1* treatment at 24 hour post infection

For the SSTF genes at 24 hour, GO and KEGG terms associated with defense responses are more enriched after △*BcSpd1* treatment. For example, GO terms about unsaturated fatty acid biosynthetic process, defense responses, kinase activity are enriched (Fig. S3A). KEGG pathways about plant-pathogen interaction, plant hormones signal transduction, fatty acid metabolism, flavonoid and isoflavonoid biosynthesis, flavone and flavonol biosynthesis are enriched (Fig. S3B). These results indicated △*BcSpd1* pretreatment affecting plant defense responses.

Next, the top 20 of KEGG enrichment are obs erved (Fig. S4A). With no surprise, plant-pathogen interaction, plant hormones signal transduction, lipid and fatty acid metabolism are on the top 5 of the KEGG enrichment. Heatmap analysis of genes enrichment in lipid and fatty acid metabolism are mostly induced and their expression levels are increased at 24 hour. In addition, many genes enriched in plant-pathogen interaction and plant hormones signal transduction are also induced by △*BcSpd1* at 24 hour (Fig. S4B-E) while other genes are kept at the higher level in CK and 14 hour. The activation of defense related genes at 24 hour would contribute to *P. ginseng* defense.

3.6. Identification and quantification of *P. ginseng* flavones upon △*BcSpd1* and *B05.10* infection

Since genes involved in flavonoid biosynthesis were enriched in △*BcSpd1*- treated ginseng at 14 hour and the leaf extraction at this

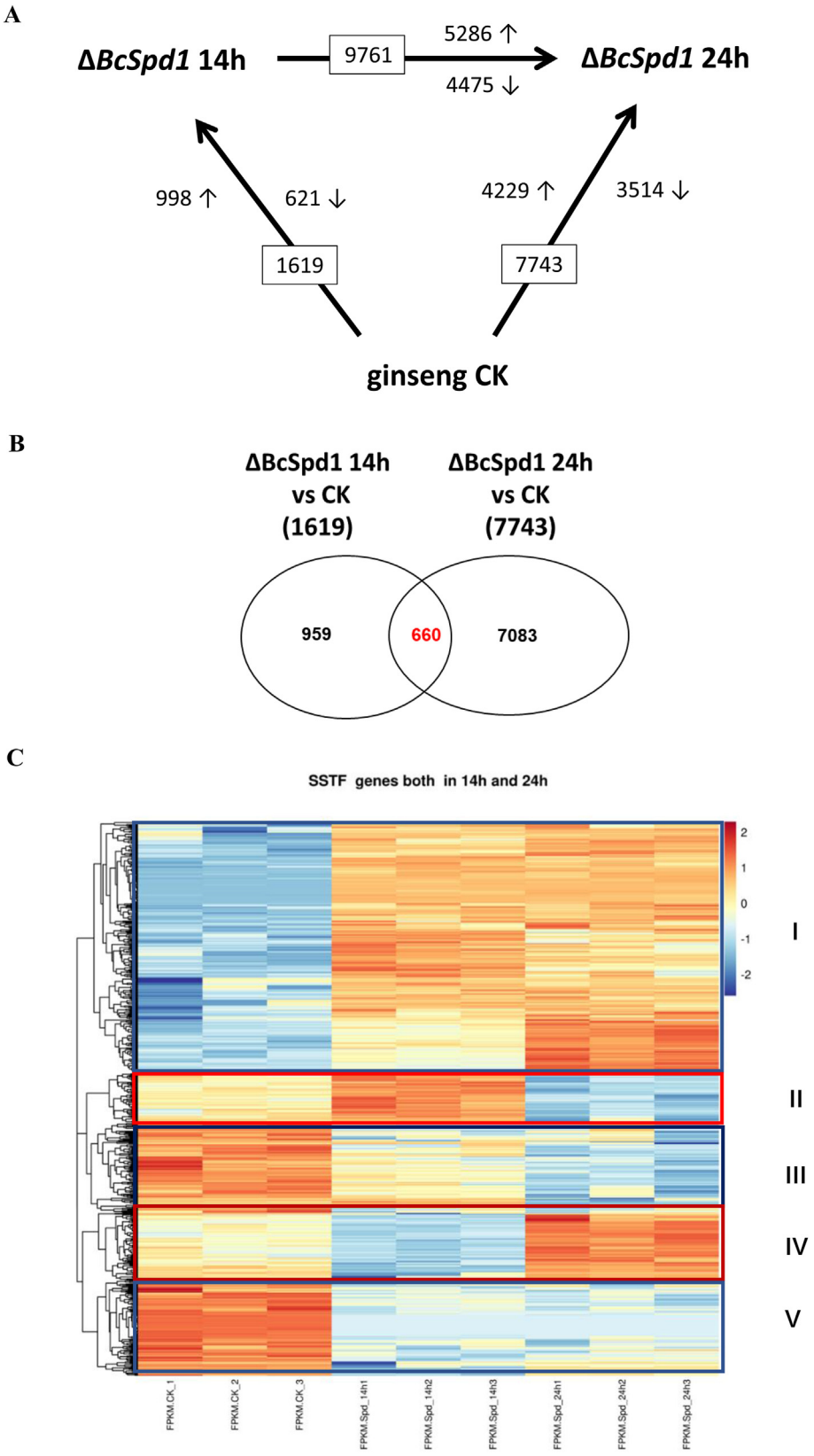

Fig. 2. Transcription analysis of *B. cinerea* Δ*BcSpd1* infected *P. ginseng* plants. (A) Number of differentially expressed genes (≥2-fold; P ≤ 0.05) in *P. ginseng* at 14h and 24h after mock treatment (CK) and Δ*BcSpd1* spray inoculation. Indicated are total number of genes between treatments or fungal treated at different time. (B) Vene analysis of the overlap genes between 14h and 24h Δ*BcSpd1* affected differentially regulated genes. (C) Heatmap analysis of differentially expressed genes observed both in CK and Δ*BcSpd1* treated ginseng plants.

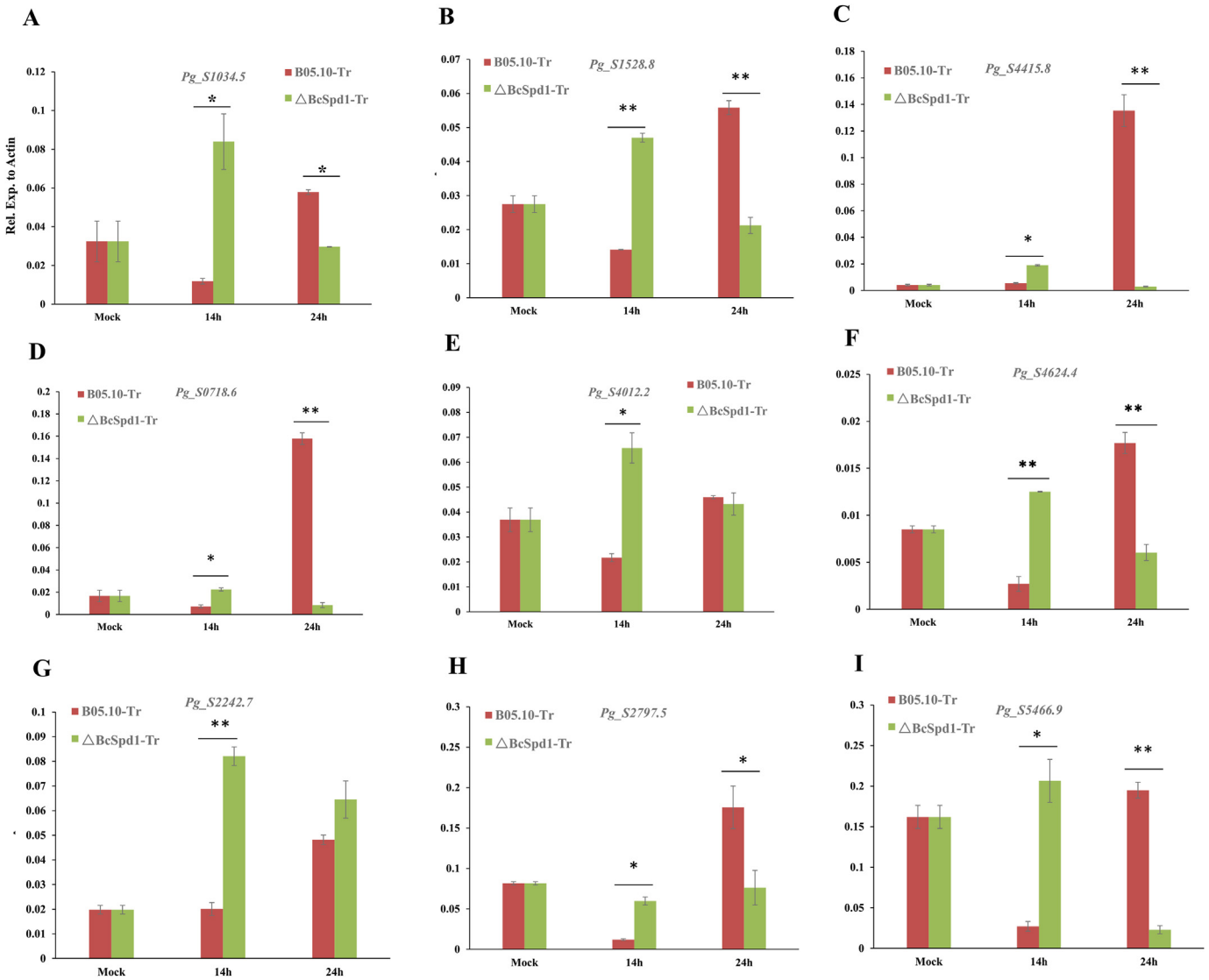

Fig. 3. Analysis of differentially expressed genes involved in ginseng defense upon *B. cinerea* Δ*BcSpd1* (Δ*BcSpd1*-Tr) treatment compared with B05.10 (B05.10-Tr). *Pg_S1034.5*: MYB-related protein 308-like; *Pg_S1528.8*: transcription factor MYC2 like; *Pg_S4415.8*: MYB -related protein Myb4-like; *Pg_S0718.6*: AP2-like ethylene-responsive transcription factor; *Pg_S4012.2*: MYB family transcription factor APL-like isoform; *Pg_S4624.4*: ethylene-responsive transcription factor ERF53-like; *Pg_S2242.7*: leucoanthocyanidin dioxygenase-like; *Pg_S2797.5*: naringenin,2-oxoglutarate 3-dioxygenase; *Pg_S5466.9*: phenylcoumaran benzylic ether reductase. Relative gene transcript levels analyzed by qPCR in ginseng and all data were normalized to ginseng *Actin*. Error bars represent SD of three biological replicates. Asterisks indicate significant differences between B05.10 or Δ*BcSpd1*-treated (*, $P < 0.05$; **$P < 0.01$, two-tailed t-test).

stage have stronger antifungal activities, we next analyzed if ginseng secondary metabolites in flavone and flavonol biosynthesis pathway, isoflavonoid biosynthesis are affected upon *B. cinerea* B05.10 and △*BcSpd1* infection.

Two-years old ginseng was sprayed with *B. cinerea* B05.10 and △*BcSpd1* for 14 hours and harvested for chemical analysis. The plants without fungi treatment were used for control. 17 metabolites in flavonoids pathway were detected in ginseng leaves under this condition. The heatmap of differentially accumulated compounds in flavonoids pathway was shown in Fig. S5. At least two groups of differentially accumulated compounds were observed (framed). Group I indicated the decreased chemicals in ginseng after *B. cinerea* B05.10 infection compared with control and △*BcSpd1* treated plants. Group II indicated the increased chemicals in ginseng upon *B. cinerea* B05.10 infection compared with control and △*BcSpd1* treated plants. The increase and the decrease of ginseng compounds might due to the differentially expressed genes upon B.05.10 and △*BcSpd1* treatments (Fig. 3).

As a detail, the naringenin, naringin, luteolin-7-O-glucoside, p-coumaric acid and phenylalanine were significantly increased in B05.10- treated ginseng compared with control (Fig. 5A,D,E,G,H) while the glycitin was significantly decreased in B05.10 than control (Fig. 5C). However, the formononetin was not detected upon B05.10 treatment compared with CK and △*BcSpd1*. In addition, the myricetin and p-coumaric acid were significantly induced in △*BcSpd1*-treated plants compared with control (Fig. 5A and B) while the concentration of naringin and epicatechin decreased (Fig. 5D,I). When compared the significantly differentially accumulated flavonoids between △*BcSpd1* and B05.10, eleven compounds were observed (Fig. 5, Fig. S6). The accumulation of glycitin, myricetin, chrysin and formononetin were higher in △*BcSpd1*- treated ginseng than B05.10- treated plants while the accumulation of naringenin, naringin, luteolin-7-O-glucoside, p-coumaric acid,

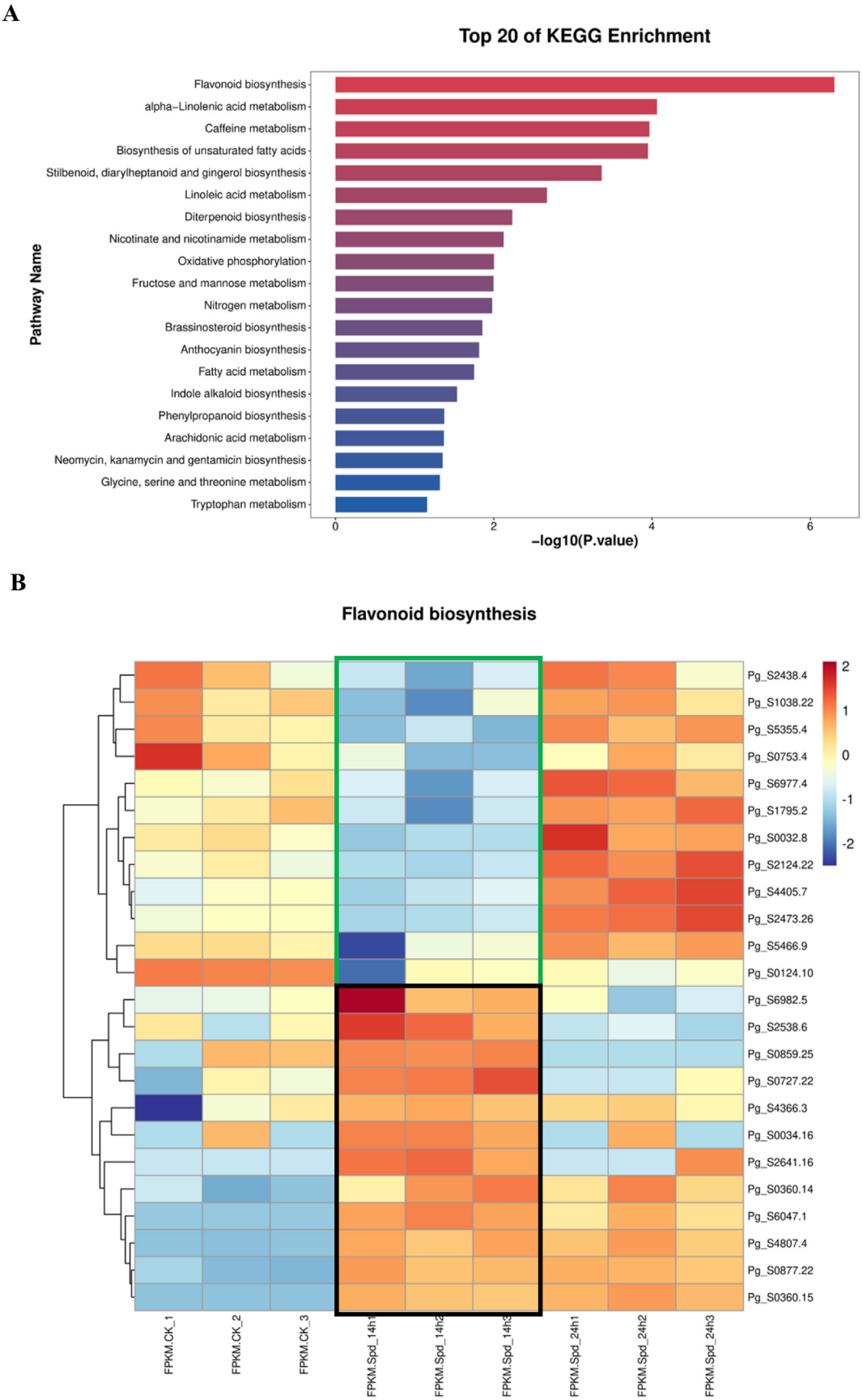

Fig. 4. Pathway analysis of differentially expressed genes at early stage upon Δ*BcSpd1* treatment. (A) Top 20 of KEGG enrichment of differentially expressed genes at 14 hours upon Δ*BcSpd1* treatment. (B) Heatmap analysis of differentially expressed genes in flavonoid pathway observed in CK, Δ*BcSpd1* treated ginseng plants at 14 hour and 24 hours respectively.

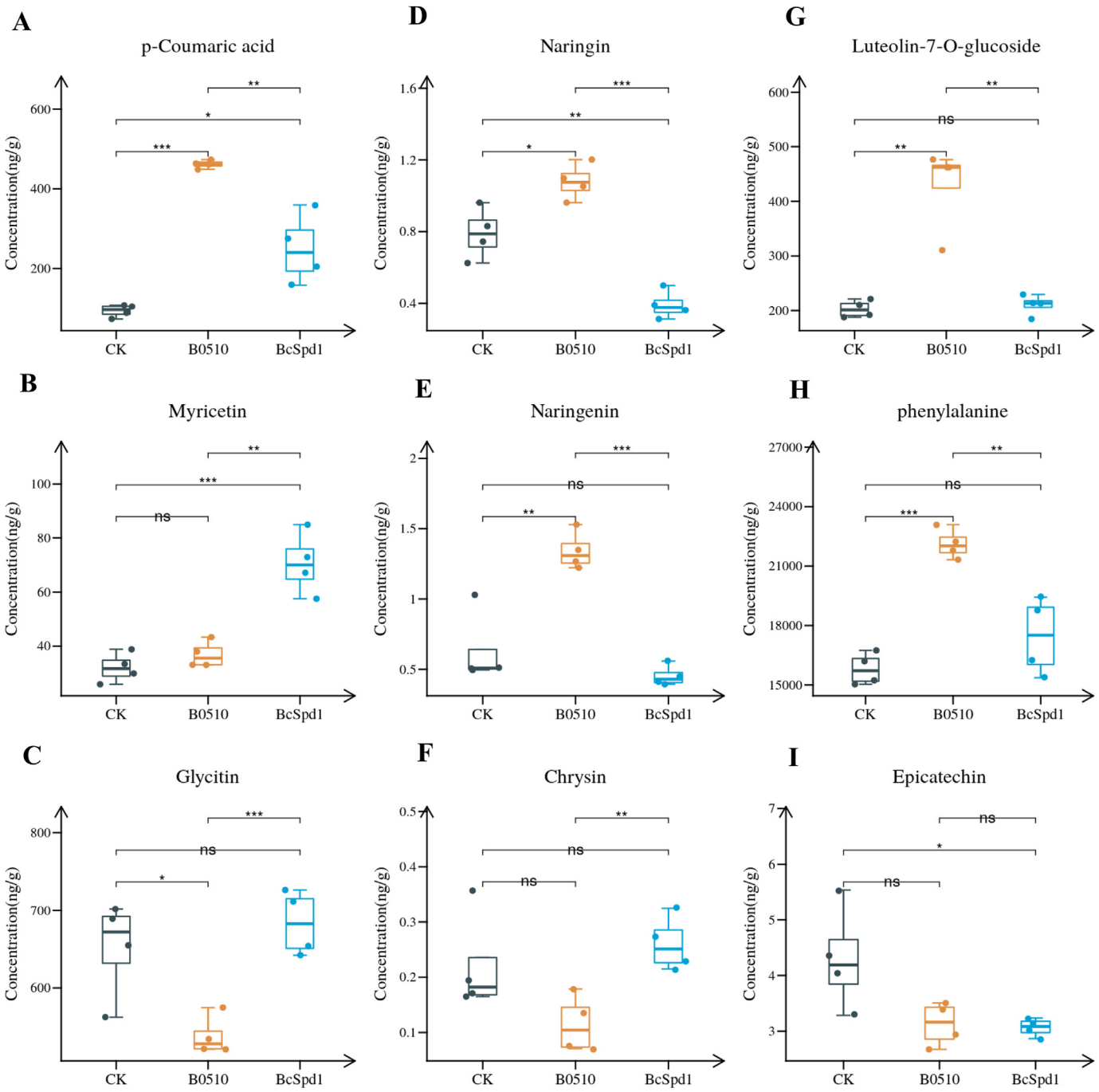

Fig. 5. Differentially accumulated chemicals in ginseng plant associated with flavonoid pathway upon *B. cinerea* B05.10 and Δ*BcSpd1* treatment at early stage. (A-I) Differentially accumulated compounds in CK, B05.10 treated ginseng and Δ*BcSpd1* treated ginseng including p-Coumaric acid (A), myricetin (B), glycitin (C), naringin (D), Naringenin (E), Chrysin (F), Luteolin-7-O-glucoside (G), Phenylalanine (H) and Epicatechin (I). Four replicates were performed and the significance between different treatments were indicated as * P < 0.05; **P < 0.01, ***P < 0.001.

phenylalanine, catechin and puerarin were lower in the former. The differentially accumulated flavonoids suggested they played a role in ginseng - *B. cinerea* interaction.

3.7. Ginseng compounds in flavonoid pathway inhibited B05.10 growth under certain concentration

Since flavonoid involved in ginseng - *B. cinerea* interaction, we next want to know if △*BcSpd1*- mediated ginseng defense responses associated with certain flavonoid compounds. As shown in Fig. 5, two compounds, p-coumaric acid and myricetin, were significantly increased in ginseng upon △*BcSpd1* treatment compared with control (Fig. 5A and B). In △*BcSpd1* treatment ginseng, the concentration of p-coumaric acid and myricetin were about 210 and 70 ng/g in average, respectively, with the ratio about 3:1. The anti-fungal activity was then performed (Fig. 6). As indicated in Fig. 6A and B, at both 24 hour and 48 hour, p-coumaric acid could significantly inhibit *B. cinerea* B05.10 growth while the inhibition rate of myricetin was lower. We also used the mixture of p-coumaric acid and myricetin under the 1:3 condition to imitate the

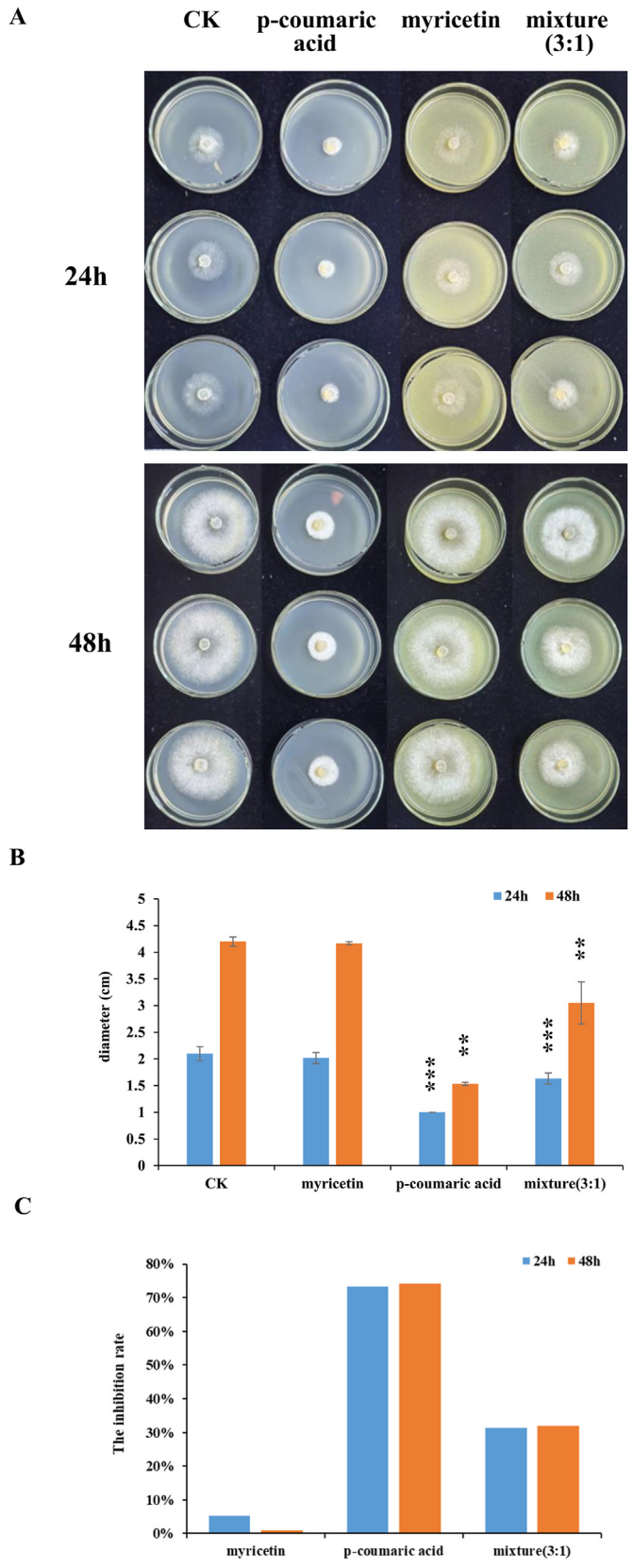

Fig. 6. Anti-fungi activity of indicated flavonoids towards *B. cinerea* B05.10. (A) Mycelial plugs of the B05.10 was inoculated on PDA medium with different compounds including p-coumaric acid, myricetin and their mixture (myricetin:p-coumaric acid = 3:1). The growth of the fungi was observed and photographed 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.

accumulation in △*BcSpd1*-treated ginseng plants and get a significant inhibition towards B05.10 either (Fig. S7). Interestingly, p-coumaric acid and its mixture with myricetin could also significantly inhibit the growth of △*BcSpd1* at both 24 a nd 48 hour, while the inhibition rate at 48 hour was lower than that on B05.10 (Fig. 6C; Fig. S7B). Thus, pretreated ginseng leaves with △*BcSpd1* would induce plant accumulation of p-coumaric acid and myricetin, which have higher toxic activity towards the virulent fungal strain B05.10, suggesting △*BcSpd1* was the good candidate fungal strain for biological application.

4. Discussion

Severe economic losses to plants are experienced globally each year due to plant diseases caused by pathogens resulting in a loss of severe yield and quality [30]. Plants are surrounded by a plethora of microorganisms, including plant growth-promoting rhizobacteria and nonpathogenic fungal strains [20,22,31–33]. Such microbes are known to exert their beneficial effects by helping plants in absorption of water, nutrients and defending against the harmful microorganisms [22,31,32]. Now, the agroecosystems have been unbalanced although the yields are improved in the last several decades [34]. Increasing public understanding of these issues has stimulated interests in research into the use of biocontrol strategy for crop disease management [35]. Here we used a virulence-deficient mutant of *B. cinerea* to protect the medicinal plant ginseng against this pathogen. Compared with *B. cinerea* B05.10, △*BcSpd1* reduced fungal virulence and enhanced ginseng defense. Similarly, △*BcSpd1* also lost virulence towards *A. thaliana* [27]. Thus, △*BcSpd1* was thought as avirulent fungal strain that could act as biocontrol microbe and played a role in plant disease management.

Generally, the mechanism of biocontrol microbes is mediated by performing antagonistic action on pests. It includes competition for nutrients and niches, and biosynthesis of antifungal metabolites (AFMs) [36,37]. For example, *Rhizobacteria* had antifungal activity against phytopathogens [36,37]. Most of the *Pseudomonas* biocontrol strains produced AFMs, which inhibit the growth of phytopathogenic fungi [38,39]. Rhizospheric *Serratia* sp. Produced prodigiosin pigment with highly promising antifungal mechanisms [36]. Since △*BcSpd1* was one of the gene mutants of *B. cinerea* and generated from the wild-type B05.10, the effects of suppressing pathogen could in different way.

In addition, along with their direct effects, the microbes have been shown to trigger induced defense responses [22]. For example, *Bacillus amyloliquefaciens* SN13 is a biocontrol agent for *Rhizoctonia solani* by increasing tolerance through enhanced plant protection response [31]. Some nonpathogenic fungal strains found to induce ISR in crop plants include mycorrhiza, *Trichoderma* sp., *Penicillium* sp., etc [22]. In cucumber, the initiation of ISR has also been attributed to infection caused by pathogens such as *F. oxysporum*, *Colletotrichum orbiculare*, and *Cladosporium cucumerinum* [40]. Thus, plant induced resistance could be triggered by multiple microbes [22,31,40]. Different with previous reports [36–39], here the pathogenic gene mutant strain *B. cinerea* △*BcSpd1* could also trigger plant defense response and indirectly suppressed pathogen infection. Similar to our work, the *Sclerotinia sclerotiorum* hypovirulent strain DT-8 provides protection against Fusarium head blight, stripe rust, and rice blast in wheat, rice, barley, maize, and oat, respectively [41]. *S. sclerotiorum* DT-8 was hypovirulent strain

attacked by a DNA mycovirus and acted as a biocontrol agent in different plants where they can be utilized as beneficial microorganisms [41].

The biocontrol fungi have been shown to trigger defense responses via multiple defense pathways [5,42]. The potential of *Trichoderma* species as biological plant protection agents was described almost for one century [43]. A *T. lignorum* strain was first reported to protect citrus seedlings against the *R. solani* through necrotrophic mycoparasitism the pathogen [43,44]. Plant resists to pathogen for further infection and restricts pathogen establishment by increasing the activities of hydrolases and producing lignin [45,46]. The colonized plants display hormone biosynthesis and signaling transduction, sustained elicitor maintenance, secondary metabolite production, biosynthesis of reactive oxygen species (ROS) and ROS scavengers [47]. For example, *Enterobacter asburiae* BQ9 mediated plant resistance by increasing the expression of defense related genes and antioxidant enzymes [48]. *Peanibacillus lentimorbus* B30488 inoculation of the soil decreased the accumulation of virus in *Nicotiana tabacum* by inducing stress related gene expression and antioxidant enzyme activity [49]. The inoculated plants demonstrated modulation of the ET pathway and antioxidant enzyme activity which corroborated systemic tolerance in plant [50]. Here, *B. cinerea* △*BcSpd1* treatment promotes ginseng transcription reprogramming. Around 16542 genes were significantly differentially expressed, which was about one-third of total mapped genes in ginseng, suggesting a modulating role of △*BcSpd1* in triggering plant defense. Specially, most genes involved in plant-pathogen interaction, plant hormones signal transduction which played key role in plant defense are enrichment and induced at 24 hours after △*BcSpd1* treatment. The activation of defense related genes by △*BcSpd1* would contribute to *P. ginseng* defense, those are similar with other beneficial microbes in activating plant resistance.

Previous works indicated plant immunity towards *B. cinerea* relies on the early induction of defense related genes such as *WRKY33* and *PAD3*, thereby quicky activated WRKY33-mediated downstream signals and highly accumulated the anti-fungal camalexin [28,51]. Here, enrichment of genes in flavonoid biosynthesis pathway at the early stage suggests flavonoids playing a key role in △*BcSpd1* mediated ginseng defense response. Compared with B05.10, △*BcSpd1*- treated *P. ginseng* altered genes expression in plant defense responses including genes encoding TFs, genes in JA/ET signaling and genes involved in flavonoid biosynthesis pathway, which would contribute to plants resistance and secondary metabolites changes. Indeed, △*BcSpd1*- treated *P. ginseng* altered the biosynthesis of flavonoids and was more resistance towards *B. cnirea* B05.10.

This work and other study revealed certain flavonoids could suppress *B. cinerea* growth such as myricetin, p-coumaric acid, quercetin and kaempferol [15]. Thus, flavonoids involved in ginseng defense to *B. cinerea*. Our work indicated △*BcSpd1* inducing ginseng defense very likely through modulating plant flavonoids pathway, perhaps at the early stage. Similarly, other works indicate that plants contain diverse mixtures and high concentrations of anti-fungal compounds, some of which provide the plant with basic or induced resistance against fungal pathogens and insects [52,53]. The changes in flavonoids have been isolated from various species [15,53–55]. These compounds are not only part of the innate defense response, but their production is also induced in the host's response to fungal pathogen attack [56,57]. Metabolites analysis of ginseng leaves indicates many of secondary metabolites in isoflavonoid biosynthesis, flavone and flavonol biosynthesis pathway are affected by *B. cinerea* B05.10 [15]. In addition, some flavonols have been reported to have cytotoxic and antimicrobial activity [58,59]. The quercetin and cyanidin aglycones were also shown to inhibit *C. gloeosporioides* hyphal growth and conidial germination [60,61]. Thus, with respect to their role in defense, here the potential application of fungal virulence-deficient strains including △*BcSpd1* in controlling plant diseases were observed. Candidate signaling molecules, also known as elicitors would be identified in future, particularly from △*BcSpd1*, and shown to protect the plants from pathogens.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jgr.2023.08.005.

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