Transcriptome analyses of the ginseng root rot pathogens *Cylindrocarpon destructans* and *Fusarium solani* to identify radicicol resistance mechanisms

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**ABSTRACT**

**Background:** The ascomycete fungi *Cylindrocarpon destructans* (Cd) and *Fusarium solani* (Fs) cause ginseng root rot and significantly reduce the quality and yield of ginseng. Cd produces the secondary metabolite radicicol, which targets the molecular chaperone Hsp90. Fs is resistant to radicicol, whereas other fungal genera associated with ginseng disease are sensitive to it. Radicicol resistance mechanisms have not yet been elucidated.

**Methods:** Transcriptome analyses of Fs and Cd mycelia treated with or without radicicol were conducted using RNA-seq. All of the differentially expressed genes (DEGs) were functionally annotated using the *Fusarium graminearum* transcript database. In addition, deletions of two transporter genes identified by RNA-seq were created to confirm their contributions to radicicol resistance.

**Results:** Treatment with radicicol resulted in upregulation of chitin synthase and cell wall integrity genes in Fs and upregulation of nicotinamide adenine dinucleotide dehydrogenase and sugar transporter genes in Cd. Genes encoding an ATP-binding cassette transporter, an aflatoxin efflux pump, ammonium permease 1 (*mep1*), and nitrilase were differentially expressed in both Fs and Cd. Among these four genes, only the ABC transporter was upregulated in both Fs and Cd, whereas nitrilase was downregulated in both Fs and Cd.

**Conclusion:** The transcriptome analyses suggested radicicol resistance pathways, and deletions of the transporter genes indicated that they contribute to radicicol resistance.

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1 Introduction

*Panax ginseng*, commonly known as ginseng, is an important medicinal plant that is distributed widely, especially in East Asia, including Korea, China, and Japan. In Korea, ginseng is typically cultivated in the same field for 4–6 years and is often threatened by many soil-borne fungal pathogens, including *Fusarium solani* (Fs), *Rhi-doctonia solani*, *Pythium ultimum*, and *Cylindrocarpon destructans* (Cd) [1–4]. Of these pathogens, Cd is the major cause of ginseng root rot disease, which leads to the most severe losses during ginseng cultivation [5].

In addition to causing root rot in growing ginseng, Cd (teleomorph *Nectria/Neonectria radicicola*) leads to replant failure because the fungus can survive in the soil for more than a decade after ginseng harvest. Cd can also cause root rot in other plants, including conifers and fruit trees [6,7]. Although Cd grows slower than other soil-borne fungi, it is often dominant in ginseng fields, perhaps due to its production of the secondary metabolite radicicol (Fig. 1A) [8,9].
Radicicol is an antifungal and antibiotic that inhibits signal transduction proteins, including the ATPase activity of Hsp90, which is necessary for proteins to fold and function properly [10,11]. By interacting with Hsp90, radicicol accelerates the dissociation of the Raf/Hsp90 complex, leading to inhibition of the Ras/MAP kinase signal transduction pathway [12]. Moreover, radicicol suppresses inducible nitric oxide synthase (iNOS) gene expression by blocking p38 kinase signaling [13]. p38 kinase induces iNOS activity via the lipopolysaccharide-induced signal transduction pathway [14,15]. Suppression of iNOS leads to defects in the regulation of germination, the response to heat stress, and nitrogen uptake [16–19].

Radicicol contributes to the survival of Cd and allows it to outcompete other microbes in the soil. Previously, we showed that radicicol inhibits the vegetative growth and spore germination of many fungal pathogens and soil-dwelling saprophytic fungi [3]. Interestingly, Fs is not affected by radicicol, indicating that Fs carries the mechanism for radicicol resistance and coexists with Cd in nature.

Fs, also known as Nectria haematococca, is a widespread soil-borne fungus, and nearly a century ago, it was shown that Fs associates with ginseng root rot [20]. Fs is very close phylogenetically to Cd, and it is not easy to distinguish the two species due to their morphological similarity [21,22]. The morphological and phylogenetic similarity of these two pathogens led us to hypothesize that they share similar mechanisms of radicicol resistance. In this study, we performed transcriptome analyses of Fs and Cd to identify differentially expressed genes (DEGs) after radicicol treatment. Our transcriptome analyses identified putative pathways of radicicol resistance, and our genetic analyses confirmed that two transporter genes are involved in radicicol resistance.

### 2. Materials and methods

#### 2.1. Fungal strains and media

The fungal strains *F. solani* 13chu01-05 (Fs) and *F. destructans* KACC41077 (Cd) were used for all of the experiments and were stored in 20% glycerol at −80°C. Media, including minimal medium (MM), complete medium (CM), potato dextrose agar, and carboxyl methyl cellulose, were prepared as described in The *Fusarium* laboratory manual [23].

#### 2.2. Targeted gene deletion and quantitative real-time PCR

The mutants Δ*fgAfla* and Δ*fgAbc* containing deletions of the *FGSG_09595* and *FGSG_04580* genes, respectively, were generated from *F. graminearum* GZ3639 (Fg) as previously described [24]. In brief, the 5' and 3' flanking regions of each target gene were amplified from the wild-type strain using the primer pairs Del-5'S/Del-5'R and Del-3'S/Del-3'R. A hygromycin-resistance cassette (HYG) was amplified from pIGPAPA using the primer pairs HYG-F and HYG-R. The three amplicons were then mixed and fused by polymerase chain reaction (PCR). The final structures for transformation were amplified during a third PCR step using the nested primers nestedF, nestedR, HYG nestedF, and HYG nestedR. Quantitative real-time PCR (qRT-PCR) was performed to validate the expression levels of the target gene in the wild-type strain and deletion mutants. The synthesized cDNA samples from each fungal strain were diluted to 10 ng/μl using distilled water, and 2 μl of cDNA was used for qRT-PCR. The relative transcription levels were normalized by reference gene, cyclophilin (CYP). All of the PCR primers used in this study are listed in Table S1.

#### 2.3. Radicicol resistance test

The radicicol resistance test was performed as previously described [25] with a slight modification. In brief, all of the fungal strains except Cd were cultivated on CM at 25°C for 5 days, whereas Cd was cultivated on CM at 20°C for 5 days. CM agar blocks with freshly grown mycelia were transferred to MM supplemented with 50 mg/L of radicicol. Growth of the mycelia was measured every 2 days. MM containing 5% (v/v) methanol was used as the control, and the experiment was performed three independent times. Differences between mean values for vegetative growth were determined using the post hoc Tukey test in the statistical software R version 3.5.1.

#### 2.4. RNA extraction and sequencing

Fs and Cd were cultivated in potato dextrose agar for 5 days at 25°C and 20°C, respectively. Then, each strain was inoculated into MM liquid with constant shaking at 200 rpm at 25°C or 20°C. After 7 days, mycelia were harvested by centrifugation and transferred to fresh MM supplemented with or without 50 mg/L of radicicol. After an additional 24 hour of incubation, total RNA was extracted using the easy-spin Total RNA Extraction kit (iNIRON Biotechnology, Gyeonggi-do, Korea) following the manufacturer’s protocol. Whole transcriptomes of Fs were sequenced using Illumina HiSeq4000 at Macrogen (Seoul, Korea), and whole transcriptomes of Cd were sequenced using Illumina HiSeq2500 at the National Instrumentation Center for Environmental Management (Seoul, Korea).

#### 2.5. Transcriptome and DEG analyses

The Fs reference genome sequence was downloaded from MycoCosm (https://genomes.jgi.doe.gov/fungi), and the Cd reference genome sequence was provided by Dr S.-H. Lee (Natural Institute of Horticultural and Herbal Science). RNA-seq reads from the two species were mapped to their respective reference genomes using STAR with the parameters —alignIntronMax 10000 because the default intron length setting for this program is too large. Each aligned read was made into a transcriptome assembly using Cufflinks with the options —min-
intron-length = 20 and —max-intron-length = 10000, and assemblies were merged together using Cuffmerge. Cuffdiff was used to quantify transcript abundance in terms of fragments per kilobase of transcript per million fragments mapped (FPKM) and to test the statistical significance of observed changes. DEGs were defined as genes with at least a twofold change in FPKM between the high-yield and low-yield groups, with a statistical cutoff of \( p \leq 0.05 \) and a false discovery rate of \( q < 0.001 \) [26]. Heat map was generated using heatmap.2 function of the gplots packages in R. Hierarchical clustering was performed using Euclidean measure to obtain distance matrix and complete linkage method for clustering.

2.6. Functional annotation and metabolic pathway mapping

All the assembled transcripts were subjected to the basic local alignment search tool (BLASTX) against the proteins of Fg (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000240135.3_GCF_000240135.3_ASM24013v3/GCF_000240135.3_ASM24013v3_protein.faa.gz) with an \( E \)-value < \( 10^{-5} \), and functional annotations were made based on the best hit of the BLASTX results. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping was performed based on the metabolic pathways of Fg due to the lack of metabolic pathway data for Fs and Cd. We manually integrated Fs and Cd metabolic pathway data obtained from the literature into the KEGG pathway to draw the metabolic pathways represented by the gene expression data.

3. Results

3.1. Resistance of Fg to radicicol

The structure of radicicol (Fig. 1A) is similar to the structure of zearalenone, which is produced by Fg. Both Fg and Fs were equally resistant to radicicol, whereas R. solani (Rs) was sensitive to radicicol (Fig. 1B). There was no significant difference in the vegetative growth of Fg, Fs, and Cd on radicicol-containing medium and the control medium.

3.2. Transcriptome analyses of Fs and Cd upon radicicol treatment

Transcriptome data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under accession numbers PRJNA473368 for Fs and PRJNA473390 for Cd. We mapped approximately 99.9% of the reads to the assembled transcript sequences using the STAR program. Some reads were mapped in pairs with libraries of three replicates of Fs treated without radicicol (FS), Fs treated with radicicol (RFS), Cd treated without radicicol (CD), and Cd treated with radicicol (RCD). The mapping ratio ranged from 60.8 to 61.2% with an average of 60.9% for FS and ranged from 63.2 to 64.0% with an average of 63.7% for RFS. The mapping ratio ranged from 83.1 to 83.8% with an average of 83.4% for CD and ranged from 78.1 to 82.6% with an average value of 80.0% for RCD (Table S2).

3.3. Functional annotation and DEG identification

To provide insight into the Fs and Cd transcriptome profiles with and without radicicol treatment, we analyzed the transcript levels of 3801 functionally annotated genes in Fs and 2710 functionally annotated genes in Cd using \( |\log_{2}\text{-fold-change}| \geq 2.0 \) and \( p\text{-value} \leq 0.05 \) as the cutoff criteria for DEGs (Fig. 2). Based on these criteria, we found 199 genes that were expressed differentially in Fs, including 91 upregulated DEGs and 108 downregulated DEGs. We found 91 genes that were expressed differentially in Cd, including 79 upregulated DEGs and 12 downregulated DEGs. Functional annotation using the Fg transcript database showed that genes encoding chitin synthases and genes related to cell wall integrity were upregulated in Fs, whereas genes encoding NADH dehydrogenase and sugar transporters were upregulated in Cd after radicicol treatment (Table S3).

Based on the complete functional annotation using the Fg transcript database, the Fs and Cd DEGs were analyzed as a Venn diagram using Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/). The Venn diagram divided the genes into four regions containing 66 upregulated genes in Fs, 82 downregulated genes in Fs, 38 upregulated genes in Cd, and 11 downregulated genes in Cd. Of these genes, only one was upregulated in both Fs and Cd after radicicol treatment. Two genes were downregulated in Fs but upregulated in Cd after radicicol treatment (Fig. 3; Fig. S1). Functional annotation using the Fg transcript database showed that the gene upregulated in both Fs and Cd after radicicol treatment encodes a putative ATP-binding cassette (ABC) transporter, and the two genes that were expressed differently in Fs and Cd encode aflatoxin efflux pump and ammonium permease (mep1) homologs. In addition, we found that the nitrilase gene was downregulated in both Fs and Cd (Table 1).

3.4. Functional classification of genes

For a more detailed analysis, we applied \( |\log_{2}\text{-fold-change}| \geq 1.0 \) and \( p\text{-value} \leq 0.05 \) cutoff criteria to the DEGs in Fs and Cd. This analysis divided the genes into 13 functional groups (Fig. 4). Genes involved in nucleotide metabolism, lipid metabolism, and carbohydrate metabolism showed similar expression patterns in Fs and Cd, but genes involved in translation, sorting and degradation, metabolism of complex lipids, metabolism of complex carbohydrates, metabolism of cofactors and vitamins, energy metabolism, biosynthesis of secondary metabolites, biodegradation of xenobiotics, and amino acid metabolism showed different expression patterns in Fs and Cd. Expression of biodegradation of xenobiotics and amino acid metabolism genes differed the most between Fs and Cd as their expression patterns were the opposite of one
These results suggest that Fs and Cd have both shared and unique pathways for radicicol resistance.

3.5. Influence of FgAbc and FgAfla on radicicol resistance

Transcriptome analyses showed that genes encoding a putative ABC transporter and an aflatoxin efflux pump were upregulated in Cd upon radicicol treatment. We identified the homologs of both genes using the Fg database, and we deleted each gene individually from wild-type Fg strain GZ3639. In the meantime, we observed the gene expression level of each target gene using qRT-PCR to confirm knock out of each gene (Fig. S2). The FgAbc and FgAfla mutants showed slightly reduced vegetative growth on radicicol-containing medium than on radicicol-free medium (Fig. 5), indicating that the ABC transporter and the aflatoxin efflux pump contribute to radicicol resistance although they are not major contributors to radicicol resistance.

4. Discussion

Transcriptome analyses showed that only one gene, a putative ABC transporter, was upregulated in both Fs and Cd in response to exogenous radicicol treatment, suggesting that one mechanism of radicicol resistance in Fs and Cd involves pumping radicicol out of the cytosol. Interestingly, the Cd ABC transporter is most similar to ZRA1, which is an ABC transporter involved in zearalenone production in Fg [27], and radicicol and zearalenone are structurally similar. In this study, we deleted the putative ABC transporter and aflatoxin efflux pump genes in Fg to determine whether they contributed to radicicol resistance because we cannot yet create targeted gene deletions in Fs and Cd. Vegetative growth of the FgAbc and FgAfla mutants was lower on radicicol-containing medium than on radicicol-free medium, suggesting that these genes contribute to radicicol resistance, although they are not major factors (Fig. S3). In addition, a higher level of the ABC transporter transcript was found in Fg after radicicol treatment, which supports our FgAbc growth data (Fig. S4).

In addition, RNA-seq analyses showed downregulation of a putative nitrilase gene in both Fs and Cd after radicicol treatment (Fig. S1). Nitrilase catalyzes the hydrolysis of nitriles to carboxylic acids, including the conversion of a cyano group to a carboxylic group in cyanoamino acid metabolism [28]. The KEGG pathway of cyanoamino acid metabolism shows that all amino acids go through a series of steps to produce hydrogen cyanide, which then converts to formamide and ammonium, which are involved in nitrogen metabolism (Fig. 6). Functionally, nitrilase is similar to cyanide hydratase, which converts hydrogen cyanide to formamide. Therefore, downregulation of nitrilase in Fs and Cd may influence the production of formamide, which forms the structural base of nitrogenous bases, carboxylic acids, amino acids, acyclic nucleosides, sugars, and amino sugars [29,30]. Moreover, RNA-seq analyses showed that expression of formamidase, which converts

| Commonly expressed genes in Cylindrocarpon destructans (Cd) and Fusarium solani (Fs) in response to radicicol. |
|---------------------------------------------------------------|
| **F. graminearum gene ID** | **Putative function** | **RCD/CD** | **Transcript level** | **RFS/FS** |
|---------------------------|----------------------|------------|---------------------|------------|
| FGSG_04580                | ABC transporter (ABCG, PDR) | 4.7341     | 5 × 10⁻⁵            | 2.5126     | 5 × 10⁻⁵ |
| FGSG_09595                | Aflatoxin efflux pump | 4.0339     | 5 × 10⁻⁵            | -2.3219    | 5 × 10⁻⁵ |
| FGSG_02094                | Ammonium permease 1  | 2.8911     | 5 × 10⁻⁵            | -2.4211    | 5 × 10⁻⁵ |
| FGSG_00051                | Nitrilase            | -2.5638    | 5 × 10⁻⁵            | -3.2662    | 0.0053   |

CD, Cylindrocarpon destructans treated without radicicol; FS, Fusarium solani without radicicol; RFS, F. solani treated with radicicol; RCD, C. destructans treated with radicicol.
formamide to ammonium, is altered in Fs upon radicicol treatment. These data suggest that radicicol has an inhibitory effect on cyanoamino acid metabolism, especially in the conversion of hydrogen cyanide and formamide to ammonium, and thus may affect uptake of nitrogen by Fs as occurring in a pseudomonad [31].

The mep1 gene, which encodes for a membrane transport protein, was downregulated in Fs, but upregulated in Cd after radicicol treatment (Fig. 5). Among the three types of MEPs, MEP1 and MEP3 are low affinity ammonium permeases that allow cells to grow under nitrogen-limiting conditions with ammonium present at relatively low concentrations. MEP1 expression is highest in cells grown on poor nitrogen sources and in environments with low concentrations of ammonium [32–34]. MM contains inorganic nitrate as the sole nitrogen source; thus, Fs and Cd cultivated in MM are exposed to a low concentration of ammonium. Although inorganic nitrate is a good nitrogen source for fungi, it is typically not utilized unless cells lack a preferred nitrogen source, such as ammonium [35]. Downregulation of nitrilase by radicicol may result in further ammonium deficiency. Thus, upregulation of mep1 in Cd may be a response to compensate for the low concentration of ammonium present in the environment.

Nitrogen is an essential component of nucleic acids, adenosine triphosphate, amino acids, and proteins [36]. In Cd, the only DEG involved in nitrogen metabolism and cyanoamino acid metabolism was nitrilase; however, in Fs, radicicol treatment resulted in downregulation of nitrilase and formamidase genes (Fig. 6). Moreover, downregulation of mep1 and mep3 could affect the ability of Fs to uptake nitrogen. These results may explain the...
opposing expression patterns of genes involved in amino acid metabolism in Fs and in Cd (Fig. 4).

To successfully colonize plant hosts, pathogens must prevent the accumulation of intercellular toxins and natural toxic compounds. One strategy is to pump toxic compounds out of the cell through efflux pumps [37]. The aflatoxin efflux pump belongs to the major facilitator superfamily (MFS) of membrane transport proteins and is a more selective transporter than the ABC transporter [27]. In addition, MFS transporters facilitate movement of small solutes in response to chemiosmotic gradients [38–41]. Thus, we propose two opposing reasons for upregulation of an MFS transporter after radicicol treatment in Cd: first, upregulation of an MFS transporter prevents extracellular radicicol from entering the cells, and second, radicicol can only be introduced into cells by an MFS transporter if the extracellular radicicol concentration is higher than the intracellular radicicol concentration.

Although radicicol treatment in Cd leads to downregulation of nitrilase, which contributes to nitrogen uptake, upregulation of an ABC transporter, an MFS transporter, and mep1 could compensate for this reduction in nitrogen uptake (Fig. 4); thus, Cd can both produce radicicol and protect itself from radicicol threats. Interestingly, although radicicol treatment resulted in the down-regulation of an MFS transporter, mep1, mep3, nitrilase, and formamidase in Fs, Fs still survives radicicol treatment, suggesting that Fs uses a different mechanism to resist radicicol.

Our DEG data suggest that in addition to the ABC transporter, genes involved in nucleotide synthesis were upregulated in Fs and allowed Fs to overcome the negative effects of radicicol (Fig. 4). Cells require both pyrimidines and purines for nucleotide biosynthesis [42], and they can be obtained from the preferred nitrogen source glutamine [35,43]. Glutamine can also be converted to glutamate, which is important in amino acid biosynthesis and glycolysis [44]. Our transcriptome analyses showed that expression of genes related to glutamine and glutamate metabolism were unaffected by radicicol treatment; thus, glutamine and glutamate metabolism may compensate for the negative effect of radicicol on extracellular ammonium and nitrogen uptake in Fs.

Furthermore, radicicol treatment resulted in upregulation of genes related to cell wall integrity and chitin synthases in Fs. Previous studies have shown that radicicol interacts with Hsp90, which leads to inhibition of MAP kinase signal transduction, which then results in defects in cell wall integrity [12,13,45–48]. Because chitin forms the structural base of the cell wall in fungi [49], Fs must upregulate chitin synthesis to preserve the integrity of cell wall from the negative effects of radicicol. Our DEG data suggest that the upregulation of cell wall integrity genes in response to radicicol allows Fs to survive in the presence of radicicol.

Overall, our study demonstrates that Cd and Fs respond to radicicol using both shared and unique mechanisms. The putative ABC transporter was highly upregulated in both Fs and Cd, but the MFS membrane transporter, which has high similarity to the aflatoxin efflux pump, was upregulated only in Cd. Although the expression patterns of the ABC and MFS transporters were not identical in Fs and Cd, growth of our FgAbc and FgAfla deletion mutants showed that both transporters contribute to radicicol resistance. Our RNA-seq analyses showed that radicicol affects nitrogen metabolism and uptake. The Cd transcriptome data suggest that enhancement of ammonium uptake under nitrogen-limiting conditions can be triggered by radicicol. Furthermore, the Cd transcriptome data suggest that normal glutamine and glutamate metabolism and enhancement of cell wall integrity and chitin synthase genes contribute to the survival of Fs in radicicol-containing environments. Further studies on the transmission of the radicicol signal, which induces expression of radicicol resistance genes, will facilitate elucidation of the radicicol resistance mechanisms in Fs and Cd and will provide information for ginseng disease control in the field.

Conflicts of interest

All authors have no conflicts of interest to declare.
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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.2018.11.005.

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