Comparative Metabolomics Revealed the Potential Antitumor Characteristics of Four Endophytic Fungi of Brassica rapa L.

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ABSTRACT: Endophytic fungi of medicinal plants have attracted wide attention due to their various active biochemical substances that are similar to those of the host plants and can be easily fermented and cultured. As a traditional medicine and food homologous plant in Xinjiang, Brassica rapa L. has a long history of applications. Recently, it has been shown that B. rapa L. has hypoglycemic, antimicrobial, immunomodulatory, and antioxidant properties. However, there are no studies on the function and diversity of endophytic fungi of B. rapa L. Four endophytic fungus (pr6, pr7, pr8, and pr10) strains were isolated from B. rapa L. in our laboratory. The metabolic extracts from pr10 have significant effects in terms of antitumor activity. In this study, in terms of types and contents, compared with those of the other three endophytic fungi, the dominant metabolites of pr10 were determined by comparative metabolomics analysis. The results of metabolomics analysis indicated that the metabolites of pr10 are rich in amino acids and sugar derivatives such as trehalose, whose ability to inhibit the A549 cell line has been proved. This study provides a theoretical basis for the development and utilization of B. rapa L. and its endophytic fungi to form antitumor agents.

1. INTRODUCTION

As an important part of biological resources and biodiversity in nature, endophytic fungi include bacteria, fungi, actinomycetes, and algae; are ubiquitous in plants; and spend their whole life or a period of life cycle in host plant tissues, which could not cause disease symptoms in the host plant tissues.1-2 Owing to the further study of the diversity and active metabolites of the endophytic fungi,3-5 these have attracted global attention. The results showed that endophytic fungi have rich biodiversity and can positively regulate the growth and development of the host plants.6 In addition, endophytic fungi have important biological functions such as promoting the growth of the host plants and the defense ability against biotic and abiotic stresses.1,7 Gond et al. extracted 18 kinds of endophytic fungi from the leaves of Nyctanthes arbor-tristis, a famous medicinal plant in India, and 10 kinds from the stems. They conducted mycelium inhibition tests on eight pathogenic bacteria and eight pathogenic fungi, respectively, and found that the inhibition effect on bacteria was up to 75% and on pathogenic fungi was up to 56.25%.1

Importantly, endophytic fungi can produce the same or similar physiologically active biochemical substances, which have insecticidal, antimicrobial, antitumor, immunosuppression, antioxidant, and other biological activities.5,9 Interestingly, different from the artificial chemical synthesis, such endophytic fungi can synthesize a variety of secondary metabolites and are easy to be fermented and cultured.7 Many studies have proved that endophytic fungi and their specific metabolites could enhance the defense response to both the abiotic and biotic stresses and effectively decrease the survival rate of tumor cells.10-12 Strobel et al. isolated an endophytic fungus from the stem of Tripterygium wilfordii Hook.f., which can produce a new cyclopeptide antibiotic with similar chemical properties to those of echinomycin. This cyclopeptide compound can inhibit human pathogenic fungi such as Candida albicans and Trichoderma and can be used in the treatment of fungal nail and skin diseases.11

As a traditional medicine and food homology plant, Brassica rapa L. has a long history of consumption and is favored by Xinjiang Uygur and other ethnic minorities. It has been reported that B. rapa L. contains a large amount of flavonoids, sugars and glycosides, alkaloids, volatile oils, amino acids, and other biochemical components that are beneficial for human beings.13-16 It has nonnegligible and significant value in inhibiting the mycelium growth of bacteria and fungi. Also,
with diverse endogenous metabolites and endophytic fungi, such plants could enhance the immunity of human beings in various ways. However, there are no research studies on the study of *B. rapa* L. endophytic fungi, especially on its unique metabolite fingerprint.

In this study, we isolated and identified the endophytic fungi from *B. rapa* L. and determined their antibacterial and antitumor activities with their crude metabolic extracts. Ultimately, the strain pr10, whose metabolic extracts have effective antitumor properties, was isolated from four endophytic fungi. For the endophytic fungus with good antitumor effect in *B. rapa* L., comparative metabolomics was performed here to draw a metabolism map of pr10 to elucidate the antitumor mechanism, demonstrating a unique fingerprint of active metabolites synthesized by pr10 and *B. rapa* L. This study provides a theoretical basis for the development and utilization of *B. rapa* L. in forming antitumor drugs and the rational utilization of endophytic fungi.

2. MATERIALS AND METHODS

2.1. Isolation of Endophytic Fungi and Preparation of Crude Extract. The normal and nondamaged *B. rapa* L. was rinsed with tap water and 70% ethanol for 30 s, sterile water 3 times, sodium hypochlorite solution (2.5% effective Cl⁻) for 3 min, and sterile water 4 times and dried under sterile conditions. Samples (5 g) were fully ground in a mortar containing a small amount of sterilized calcium carbonate and quartz sand mortar. Moreover, the ground samples were diluted 10 and 100 times with sterile water. The diluted smear on the Petri dishes containing potato dextrose agar (PDA) and cultured at 28 °C, away from light. The tip part of the newly formed mycelium was transferred to the new PDA medium, then purified, and cultured 5–7 times until the pure strain was obtained.

Genomic DNA was extracted from the strains, according to the manufacturer’s protocols. The internal transcribed spacer (ITS) region of rDNA was amplified using primers ITS1F and ITS4. The sequences were compared with those available in GenBank via BLAST. Phylogenetic analysis was conducted using the neighbor-joining method in MEGA5.

The endophytic strain was inoculated into the Erlenmeyer flask containing potato dextrose broth (PDB) medium and fermented at 28 °C for 15 days. Then, the culture medium was extracted by organic solvents ethyl acetate (EtAc) 3 times. After that, all extracts were evaporated in a rotary evaporator under reduced pressure.

2.2. Antitumor Activity Assay Determination (CCK8). Human alveolar adenocarcinoma cells (AS49) in the logarithmic growth phase with good growth state were taken, the cell density was adjusted to $5 \times 10^4$ mL⁻¹ by Dulbecco’s modified Eagle’s medium (DMEM), and the cell suspension of 100 microcells per well was added into a 96-well plate. At the same time, the PBS blank group and normal cell control group were set for overnight culture at 37 °C (100 microcells were added into the holes around cell holes). Cells were treated respectively according to different groups and cell treatment settings. The extract (200 μg/mL), which is diluted by the medium, was added to the cells in the treatment group, PBS in the blank group, and no addition in the control group. Each group was cultured for 48 h in an incubator with 5% CO₂ and 37 °C. CCK8 (10 μL) was added to each well and cultured at 37 °C for 4 h. The absorbance OD₅₇₀ was determined by a microplate reader.

2.3. Sample Preparation and Extraction for Metabolomics Analysis. The four endophytic fungi were expanded on PDA and cultured in PDB. Each fungus was fermented for 15 days at 180 rpm and 28 °C, and each fungus has six replicates. Mycelia and cell structure were broken by an ultrasonic instrument, then the organic products were extracted by ethyl acetate with an equal proportion 3 times, the organic products were rotation-dried at 45 °C, and the coarse metabolites of fungi were obtained by vacuum-drying in an oven. All of the samples were prepared for metabolomics analysis.

2.4. Gas Chromatography–Time-of-Flight Mass Spectrometry (GC-TOF-MS) Analysis. GC-TOF-MS analysis was performed using an Agilent 7890 gas chromatograph coupled with a time-of-flight mass spectrometer. The system utilized a DB-SMS capillary column. An aliquot (1 μL) of the sample was injected in splitless mode. Helium was used as the carrier gas, the front inlet purge flow was 3 mL min⁻¹, and the gas flow rate through the column was 1 mL min⁻¹. The initial temperature was kept at 50 °C for 1 min, then increased to 310 °C at a rate of 10 °C min⁻¹, and then kept for 8 min at 310 °C. The injection, transfer line, and ion source temperatures were 280, 280, and 250 °C, respectively. The energy was 70 eV in electron impact mode. The mass spectrometry data were acquired in full-scan mode with the m/z range of 50–500 at a rate of 12.5 spectra/s after a solvent delay of 6.33 min.

2.5. Raw Data Preprocessing. The original data included eight quality control (QC) samples and 24 experimental samples; ultimately, 296 peaks were extracted from the raw data profile. To better analyze the data, a series of preparations and arrangements were performed based on the original data. The noise was removed by filtering individual peaks. Also, deviation values were filtered based on the interquartile range and individual peaks were further filtered. Only the peak area data with a null value not more than 50% in a single group or a hollow value not more than 50% in all groups were retained. The missing value recoding in the original data was simulated by 1/2 of the minimum value. Moreover, the data were further normalized by an internal standard (IS). Finally, 275 peaks were preserved after preprocessing and all peaks in this study were searched and identified in the local database by its MS/MS information.

2.6. Statistical Analysis. Metabolites have been used for hierarchical clustering analysis (HCA), principle component analysis (PCA), and partial least squares-discriminant analysis (OPLS-DA) by R (www.r-project.org/) to study metabolite cultivar-specific accumulation, according to the normalized peak area of metabolites. The main analytical parameters of P-value and fold change were 0.05 and 2.0, respectively.

To further determine the biological significance associated with antitumor activity, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to link differential metabolites to metabolic pathways in pr10 compared with other three endophytic fungi (pr6, pr7, and pr8). Enrichment P-values were computed from a hypergeometric distribution. A P-value of <0.05 was selected to reduce the false discovery rate.

3. RESULTS

3.1. Antitumor Activity of Endophytic Fungal Metabolites. Totally, four endophytic fungi were isolated and used to determine its antitumor activity. Antitumor activity of ethyl acetate extract of fungi (pr10, pr6, pr7, and pr8) against human alveolar adenocarcinoma cell line AS49 was
determined by CCK8 assays. The result shows that the 200 μg/mL concentration extract produce more than 50% reduction in viability of A549 cells and its inhibition on other cell lines is also better than those of pr6, pr7, and pr8 (Table 1). However, pr6, pr7, and pr8 did not show significant inhibition of A549 cells and other cell lines. The phylogenesis suggests that pr10 is a member of Alternaria. It has the closest genetic relationship with Alternaria brassicae. The mycelium morphology also indicated that the hypha of pr10 has the characteristics of Alternaria such that the hyphae are septate and brown conidiophores are solitary or clustered (Figure 1).

Table 1. Antitumor Activity of Extraction from Four Endophytic Fungi

| name | AS49 (mean±SD) | blank | control | inhibition ratio (%) |
|------|----------------|-------|---------|----------------------|
| pr10 | 0.584 ± 0.011** | 0.206 ± 0.003 | 1.055 ± 0.012 | 55 |
| pr6  | 1.015 ± 0.013B* | 0.206 ± 0.003 | 1.055 ± 0.012 | 5 |
| pr7  | 0.860 ± 0.011  | 0.206 ± 0.003 | 1.055 ± 0.012 | 23 |
| pr8  | 0.963 ± 0.011  | 0.206 ± 0.003 | 1.055 ± 0.012 | 11 |

**P < 0.01, *P < 0.05, blank represents the absorption values of pbs, and the control represents the value of A549 cell lines without treatment.

Figure 1. Phylogenetic identification and mycelial morphology of pr10. (A) Phylogenetic analysis of pr10 by its ITS gene sequence. pr10 and A. brassicae are used as a group. (B) Mycelial morphology of pr10 by an optical microscope (40×).

Figure 2. Global analysis of the metabolic profile of endophytic fungi. (A) Principle component analysis indicating the distinct biological variation among all samples. The ellipses with different colors represent the replicates of each endophytic fungi (red, pr10; green, pr6; navy, pr7; and blue, pr8). Scatter colors and shapes represent experimental groupings of samples. All samples are within 95% confidence intervals (Hotelling’s T-squared ellipse). (B) Hierarchical clustering of the 24 samples used in this study showing two distinct clades: one comprised of pr10 exhibiting a significantly specific metabolic profile of pr10.
To investigate the factor that causes the effective antitumor activity of pr10 that is different with other three endophytic fungi, six biological replicates of each endophytic fungus were harvested for metabolomics analysis. For this experiment, metabolites of such four endophytic fungi cultured for 15 days were extracted. Principle component analysis (Figure 2A) was used to evaluate the biological variability among all samples and the metabolic differences among such four endophytic fungi. The result of PCA analysis indicated that four distinct regions with different colors were clustered, especially in sample pr10 that formed a red oval demonstrating that there is a significant metabolic difference between fungus pr10 and other three endophytic fungi. Therefore, with the antineoplastic activity of pr10, it could be concluded that the metabolic profile of pr10 has a potential antitumor effect.

3.2. Discovery of Candidate Metabolites from All Metabolomics Profiles. Based on the most effective antitumor activity of pr10 compared with those of other three endophytic fungi, the identified metabolites that matched the conditions (log 2 fold changed ≥ 2, FDR ≤ 0.05, and VIP > 1.0) were defined as candidate metabolites (Figure 3A−C). The volcano plot was used to excavate the candidate metabolites in each pairwise comparison group (D15-pr10 vs pr6, D15-pr10 vs pr7, and D15-pr10 vs pr8). As can be seen from the OPLS-DA score plot, the differences between each pairwise comparison group of samples are very significant and all samples are within 95% confidence intervals (Hotelling’s T-squared ellipse). Permutation test of the OPLS-DA model for each pairwise comparison group. The vertical coordinate represents the value of R2Y or Q, and the green dot shows the value of R2Y obtained by the substitution test. The blue square shows the value of Q obtained by the substitution test, and the two dotted lines represent the regression lines of R2Y and Q, respectively.

Figure 3. Excavating of candidate metabolites in each pairwise comparison. (A−C) Volcano plot showed the metabolites match the condition (log 2 fold changed ≥ 2, FDR ≤ 0.05, and VIP > 1.0) in each pairwise comparison and dug out the candidate metabolites with various increased and decreased levels. Each point in the volcano diagram represents a metabolite, and the horizontal coordinate represents the multiple changes of the group of substances compared (log 2 fold change), and the vertical coordinate represents the P-value of Student’s t-test (−log10 P-value). The red plot and blue plot represent the significantly increased and decreased metabolites, respectively. The metabolites labeled by the gray plot are not significant in a pairwise comparison. Moreover, the scatter size represents the VIP value of the OPLS-DA model; the larger the scatter point, the larger the VIP value. (a, c, e) OPLS-DA analysis of each pairwise comparison (D15-pr10 vs pr6, D15-pr10 vs pr7, and D15-pr10 vs pr8). As can be seen from the OPLS-DA score plot, the differences between each pairwise comparison group of samples are very significant and all samples are within 95% confidence intervals (Hotelling’s T-squared ellipse). (b, d, f) Permutation test of the OPLS-DA model for each pairwise comparison group. The vertical coordinate represents the value of R²Y or Q, and the green dot shows the value of R²Y obtained by the substitution test. The blue square shows the value of Q obtained by the substitution test, and the two dotted lines represent the regression lines of R²Y and Q, respectively.

pr10 formed a separate clade, demonstrating that there is a significant metabolic difference between pr10 and other three endophytic fungi. Therefore, with the antineoplastic activity of pr10, it could be concluded that the metabolic profile of pr10 has a potential antitumor effect.
against a local database with its MS/MS fragment information (Figure 3A). After comparing pr7, 47 metabolites were increased in pr10 with various levels and 8 out of all identified metabolites were unknown. Finally, compared with pr8, 46 metabolites were in a dominant position and 9 out of all different metabolites were unknown.

To clearly analyze the interference degree of the antitumor effect of pr10, the OPLS model was utilized. The OPLS score plot (Figure 3a,c,e) at each point indicated a sample, and each clustering represented a corresponding metabolic pattern in six different groups. Obvious separation of all pairwise comparison groups (D15-pr10 vs pr6, D15-pr10 vs pr7, and D15-pr10 vs pr8) was observed, and the R2Y values of the permutation test...
of the OPLS-DA model for each pairwise comparison group were 0.75 (D15-pr10 vs pr6), 0.87 (D15-pr10 vs pr7), and 0.86 (D15-pr10 vs pr8) ($P \leq 0.05$). The original model $R^2_Y$ is very close to 1, indicating that the established model conforms to the real situation of sample data. The original model $Q$ value is very close to 1, indicating that if new samples are added to the model, approximate distribution will be obtained. In general, the original model can well explain the differences between the two groups of samples. The $Q$ values of the random model of the permutation test are all smaller than the $Q$ values of the original model. Moreover, in the random model, the gradually decreased $Q$ value associated with the increase in the proportion of $Y$ variable, indicating that the original model is robust and does not over fit. In addition, all these results of OPLS-DA analysis indicated high model reliability and significant difference in each pairwise comparison.

3.3. Macromaps of the Relative Content of Candidate Metabolites in Each Pairwise Comparison Group. To dig out the metabolites that caused the differences among these four endophytic fungi and identify unique and valuable compounds of fungus pr10, hierarchical clustering analysis

Figure 6. Pathway analysis of the candidate metabolites from D15-pr10 vs pr7. The results of pathway enrichment analysis of the candidate metabolites identified from D15-pr10 vs pr7 are shown in the bubble diagram, and each bubble means a metabolic pathway enriched in the pairwise comparison group.

Figure 7. Pathway analysis of the candidate metabolites from D15-pr10 vs pr8. Pathway analysis of the metabolites filtered in D15-pr10 vs pr8 by the conditions (log 2 fold change $\geq 1.0$, $P < 0.05$, and VIP $> 1.0$).
was further performed based on the relative content of the metabolites in each pairwise comparisons. Also, the heatmaps indicated that all of the samples could be clustered together based on the significantly increased and decreased metabolites in each pairwise comparison (Figure 4A–C). Moreover, the increased metabolites are less than the decreased metabolites in D15-pr10 vs pr6. However, the increased metabolites are significantly more than the decreased metabolites in D15-pr10 vs pr7. In addition, all of the metabolites display a stable character in each replicate of each endophytic fungi, demonstrating that it is meaningful to find some components having potential antitumor activities in the specific endophytic fungus pr10. All of the increased metabolites in pairwise comparison were isolated to perform further function analysis by pathway enrichment analysis.

3.4. Pathway Enrichment Analysis of the Candidate Metabolites in Three Pairwise Comparison Groups. To further analyze the function of the candidate metabolites, and the significantly affected pathway that produces such key metabolites, pathway enrichment analysis was performed against the KEGG database. The enrichment results of the candidate metabolites from D15-pr10 vs pr6 indicated that amino acid metabolism is the main differential metabolic pathway, such as valine, leucine, and isoleucine biosynthesis; valine, leucine, and isoleucine degradation; and aminoacyl-tRNA biosynthesis; in addition, pantothenate and CoA biosynthesis (Table 4). Finally, in the pr8 vs pr10 group, except the metabolic pathways mentioned above, there were some other metabolic pathways such as the pentose phosphate pathway, fructose and mannose metabolism, glycolysis or gluconeogenesis, and starch and sucrose metabolism. It has been reported that glycometabolism plays an important role in the antitumor activity and has potential antitumor activity.21 Other metabolites such as biochemical components produced by the biosynthesis of antibiotics (Table 2), which was enriched in all of these three pairwise comparisons and could function in defense response against biological stress, also have potential antitumor activities with their microorganism inhibiting property.

Compared with those in the other three endophytic fungi pr6, pr7, and pr8, there are 13 metabolic pathways that were significantly different and highly expressed in pr10 (Figure 8 and Tables 3–5). All 13 metabolic pathways were variously enriched in these three pairwise comparison groups; for D15-pr10 vs pr6, only two pathways were significantly enriched (e.g., valine, leucine, and isoleucine biosynthesis and β-alanine metabolism) and it had been reported that valine and leucine play an important role in a new antitumor drug that could improve the antitumor efficiency (Table 3). For group pr10 vs pr7, four biosynthesis pathways were significantly enriched (P < 0.05) (e.g., glycine, serine, and threonine metabolism; valine, leucine, and isoleucine biosynthesis; β-alanine metabolism; and aminoacyl-tRNA biosynthesis) (Table 4). Finally, in the pr8 vs pr10 group, except the metabolic pathways mentioned above, there were some other metabolic pathways such as the pentose phosphate pathway and lysine degradation pathway (Table 5). Among all 13 pathways, the metabolites synthesized in starch and sucrose metabolism were most likely to have antitumor activities with their microorganism inhibiting property.

3.5. Excavation of the Most Important and Specific Metabolites of pr10. All of the increased metabolites in pairwise comparative groups were classified in the upset plot
to excavate the specific metabolites that are higher in pr10 than in other endophytic fungi. The count of the metabolites with such a property is 20, and in pr10, all such 20 metabolites are higher than their contents in the other three endophytic fungi. Moreover, there are various amounts of the metabolites involved in the intersection, which are shown in Figure 10. All such 20 metabolites and their properties (VIP and P-value) are shown in Table 5, demonstrating a significant difference in each pairwise comparison. However, among all 20 metabolites, 9 components were still not identified with a detailed MS/MS information, which need further identification and determination of their antitumor activities.

Except for these 20 metabolites, there are other three metabolites that could function in the antitumor process such as trehalose, D-arabitol, and phenylalanine (Figure 11). For trehalose, the results indicated that, compared with that of pr7, the content of trehalose increased by 2.67-fold in pr10, demonstrating that pr10 could synthesize more trehalose in vivo (Figure 11B). It has been reported that trehalose and its derivates could perform various functions in the antitumor process. For other metabolites, D-arabitol could effectively influence the proliferation of tumor cells and angiogenesis during tumor growth and the content of such valuable metabolites is 30.65-fold more than its content in pr6. Many studies have reported that phenylalanine and its derivates could suppress the growth and survival rate of tumor cells in different ways. However, the content of phenylalanine is about 230 000-fold in pr10 more than that in the other three endophytic fungi. Moreover, all three metabolites have potential antitumor activities, contributing the antitumor activity of pr10 on A549 cell lines. Further analysis of the relative content of such 20 metabolites suggests that these metabolites could form three clades in the hierarchical clustering analysis. Also, in the upper cluster, four metabolites are identified (e.g., valine, phosphate, tagatose 1, and threonine). The contents of all of the metabolites in this cluster display a lower-level increase from about 2.2- to 200-fold than their contents in the other three endophytic fungi (Figure 10). In the bottom cluster, all of the metabolites were identified by MS/MS and such six metabolites (lysine, cellobiose, tyrosine, gentiobiose, tartaric acid, and phenylalanine) exhibit a relatively higher increase (about 8.0- to 220-fold increase) (Figure 10). Moreover, it has been proved that phenylalanine and its derivates have potential antitumor activity. In the middle cluster, only one compound, pantothenic acid, was identified (Figure 10). In pr10, the content of pantothenic acid is about 33 000-fold than its content in the other three endophytic fungi. In addition to this, the other six significantly increased metabolites were not identified (P < 0.05).

Table 3. Significance Level of Key Metabolic Pathways Filtered by the Venn Diagram in the D15-pr7 vs pr10 Group

| Pathway Name (Primer Name)                           | P-Value (P<0.05) | Pairwise Comparison (D15-pr7 vs pr10)                          | Hits (Metabolites)                           |
|-----------------------------------------------------|------------------|----------------------------------------------------------------|---------------------------------------------|
| Glycine, serine and threonine metabolism            | 0.020133         | L-Aspartic acid; L-Threonine; Pyruvic acid                     |                                             |
| Valine, leucine and isoleucine biosynthesis         | 0.0001312        | L-Threonine; L-Valine; L-Isoleucine; Pyruvic acid; 4-Methyl-2-oxopentanoate |                                             |
| Lysine biosynthesis                                 | 0.069931         | L-Aspartic acid; L-Lysine                                       |                                             |
| Glyoxylate and dicarboxylate metabolism             | 0.28363          | Succinic acid                                                  |                                             |
| beta-Alanine metabolism                             | 0.01016          | L-Aspartic acid; Pantothenic acid                              |                                             |
| Aminoacyl-tRNA biosynthesis                         | 0.0029699        | L-Aspartic acid; L-Valine; L-Lysine; L-Isoleucine; L-Threonine; L-Proline |                                             |
| Valine, leucine and isoleucine degradation          | 0.0050393        | L-Valine; L-Isoleucine; 4-Methyl-2-oxopentanoate                |                                             |
| Pantothenate and CoA biosynthesis                   | 0.0050393        | Pantothenic acid; Pyruvic acid; L-Valine                       |                                             |
| Starch and sucrose metabolism                       | 0.34942          | Trehalose                                                      |                                             |
| Arginine and proline metabolism                     | 0.21257          | L-Aspartic acid; L-Proline                                     |                                             |
| Citrate cycle (TCA cycle)                           | 0.076628         | Succinic acid; Pyruvic acid                                    | D-Ribose                                    |
| Pentose phosphate pathway                           | 0.34942          |                                                               |                                             |
| Lysine degradation                                  | 0.31728          |                                                               | L-Lysine                                     |
In the interaction process of the endophytic fungi and host plants, the host plants provide nutrition and shelter to the endophytic fungi and, in return, endophytic fungi could synthesize many effective and active biochemical components that could enhance the resistance against various biotic and abiotic stresses in the host plants. Although this has gained extensive attention all over the world, this is still in the initial stage of research in terms of the diversity and function of endophytic fungi, and such research studies could be a new addition to the available diversity of fungi. There is no research on the function and diversity of endophytic fungi of *B. rapa* L., a widely used plant with potential medicinal properties. It has been reported that *B. rapa* L. has abundant biochemical components such as flavonoids, phenolic acid, amino acids, carbohydrates, and vitamins; all of these metabolites could enhance the antioxidant ability and resistance of human beings in various aspects such as antitumor, immune regulation, biotic stress, and abiotic stress. In consideration of these, conducting research that could enhance the resistance against various biotic and abiotic stresses in the host plants.

**Table 4. Significance Level of Key Metabolic Pathways Filtered by the Venn Diagram in the D15-pr8 vs pr10 Group**

| Pathway Name (Primer Name)                                      | Pairwise Comparison (D15-pr8 vs pr10) |
|----------------------------------------------------------------|---------------------------------------|
|                                                                | P-Value (P<0.05)                      |
|                                                                | Hits (Metabolites)                     |
| Glycine, serine and threonine metabolism                      | 0.13462                               | L-Aspartic acid; L-Threonine               |
| Valine, leucine and isoleucine biosynthesis                  | 0.02041                               | L-Threonine; L-Valine; 4-Methyl-2-oxopentanoate |
| Lysine biosynthesis                                          | 0                                     | L-Aspartic acid; L-Lysine                 |
| Glyoxylate and dicarboxylate metabolism                     | 0.11232                               | Citric acid                               |
| beta-Alanine metabolism                                      | 0                                     | L-Aspartic acid; Pantothenic acid         |
| Aminoacetyl-tRNA biosynthesis                                | 0                                     | L-Aspartic acid; L-Valine; L-Lysine; L-Threonine; L-Proline |
| Valine, leucine and isoleucine degradation                   | 0                                     | L-Valine; 4-Methyl-2-oxopentanoate        |
| Pantothenate and CoA biosynthesis                            | 0.15306                               | Pantothenic acid                          |
| Starch and sucrose metabolism                                | 0.07449                               | D-Maltose                                 |
| Arginine and proline metabolism                              | 0                                     | L-Aspartic acid; L-Proline; Hydroxyproline|
| Citrate cycle (TCA cycle)                                    | 0.09869                               | Citric acid                               |
| Pentose phosphate pathway                                    | 0.03369                               | 6-Phosphogluconono-D-lactone; Gluconic acid|
| Lysine degradation                                           | 0                                     | L-Lysine                                  |

*Colors represent the significance of each metabolic pathway in the D15-pr6 vs pr10 group; red represents a highly significant difference. The metabolites involved in each pathway are shown in the Hits list.

**Table 5. Significance of Indispensable Metabolites Filtered by the Upset Diagram**

| Metabolite                  | D15-pr8 vs pr10 | D15-pr7 vs pr10 | D15-pr6 vs pr10 |
|-----------------------------|-----------------|-----------------|-----------------|
|                             | VIP (VIP > 1.0) | P-value (P < 0.05) | VIP (VIP > 1.0) | P-value (P < 0.05) | VIP (VIP > 1.0) | P-value (P < 0.05) |
| pantothenic acid            | 1.25            | 0.03            | 1.32            | 0.03            | 1.18            | 0.03            |
| threonine 1                 | 1.73            | 0.01            | 1.76            | 0.01            | 1.27            | 0.05            |
| analyte 132                 | 1.81            | 0.00            | 1.91            | 0.00            | 1.76            | 0.00            |
| lysine                      | 1.81            | 0.01            | 1.55            | 0.01            | 1.50            | 0.01            |
| gentiobiose 2               | 1.57            | 0.02            | 1.91            | 0.02            | 1.54            | 0.02            |
| phosphate                   | 1.07            | 0.00            | 1.26            | 0.00            | 1.37            | 0.00            |
| phenylalanine 2             | 1.53            | 0.01            | 1.61            | 0.01            | 1.16            | 0.03            |
| tagatose 1                  | 1.59            | 0.03            | 1.37            | 0.03            | 1.22            | 0.03            |
| analyte 197                 | 1.56            | 0.00            | 1.85            | 0.00            | 1.34            | 0.00            |
| unknown                     | 1.54            | 0.02            | 1.64            | 0.02            | 1.51            | 0.02            |
| tartaric acid               | 1.53            | 0.02            | 1.61            | 0.02            | 1.25            | 0.02            |
| analyte 173                 | 1.09            | 0.00            | 1.19            | 0.01            | 1.08            | 0.00            |
| unknown 128                 | 1.33            | 0.00            | 1.65            | 0.00            | 1.07            | 0.01            |
| cellobiose 1                | 1.50            | 0.01            | 1.37            | 0.01            | 1.15            | 0.01            |
| tyrosine 1                  | 1.81            | 0.00            | 1.91            | 0.00            | 1.48            | 0.00            |
| unknown                     | 1.80            | 0.02            | 1.90            | 0.02            | 1.76            | 0.02            |
| valine                      | 1.26            | 0.01            | 1.86            | 0.01            | 1.43            | 0.01            |
| unknown                     | 1.53            | 0.02            | 1.63            | 0.02            | 1.50            | 0.02            |
| unknown                     | 1.53            | 0.04            | 1.61            | 0.04            | 1.51            | 0.04            |
| analyte 659                 | 1.08            | 0.03            | 1.35            | 0.03            | 1.27            | 0.03            |

*VIP, variable importance in the projection (VIP > 1.0); P < 0.05 (Student’s t-test).

**Figure 9. Filtration of significantly increased metabolites of pr10.** The upset diagram analysis show 20 metabolites, which were represented by red circles and recognized as indispensable components, that were increased in these three pairwise comparison groups (D15-pr6 vs pr10, D15-pr7 vs pr10, and D15-pr8 vs pr10). The black bars display the number of metabolites involved in each intersection.

4. DISCUSSION

In the interaction process of the endophytic fungi and host plants, the host plants provide nutrition and shelter to the endophytic fungi and, in return, endophytic fungi could synthesize many effective and active biochemical components that could enhance the resistance against various biotic and abiotic stresses in the host plants. Although this has gained extensive attention all over the world, this is still in the initial stage of research in terms of the diversity and function of endophytic fungi, and such research studies could be a new addition to the available diversity of fungi. There is no research on the function and diversity of endophytic fungi of *B. rapa* L., a widely used plant with potential medicinal properties. It has been reported that *B. rapa* L. has abundant biochemical components such as flavonoids, phenolic acid, amino acids, carbohydrates, and vitamins; all of these metabolites could enhance the antioxidant ability and resistance of human beings in various aspects such as antitumor, immune regulation, biotic stress, and abiotic stress. In consideration of these, conducting research...
on the diversity and biological function of endophytic fungi of *Brassica rapa* L. is highly deserved.

In this study, four endophytic fungi were isolated from *B. rapa* L. and further purified in vitro. It has been reported that endophytic fungus extracts from the medicinal plant Umea could effectively inhibit multidrug-resistant bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* and found that 60% of the crude extracts had antibacterial effects on these bacteria (Manila). In addition, Refaei et al. isolated eight strains of endophytic fungi from Dahua, among which three strains of crude extracts from fermentation broth had antibacterial effects on *C. albicans*. To reveal its function and application value, its mycelium inhibition in vitro was tested based on its crude extracts. The result indicated that the crude extracts could not effectively inhibit the growth of pathogenic bacteria (*E. coli*, *Staphylococcus aureus*, *C. albicans*, *P. aeruginosa*, and *Enterococcus faecalis*). In addition to the antibacterial ability of endophytic fungi, it has been reported that they have antitumor functions because of their uniquely synthesized metabolites. We further tested the antitumor ability of endophytic fungi with their crude extracts; the results indicated that among all of these four endophytic fungi the crude extracts of pr10 could effectively inhibit tumor cells by 54%; however, other three crude metabolites from pr6, pr7, and pr8 did not display significant antitumor effect. For the results of antitumor effect, it is likely due to the use of metabolized crude extract from pr10, including D-Arabitol, Trehalose, and Phenylalanine. With effective antitumor metabolites, we performed a comparative metabolomics analysis to study the different metabolites among these four endophytic fungi from species to contents, especially the metabolites from pr10.

For the metabolites in all endophytic fungi, there are 296 metabolites being acquired; and among all such acquired metabolites, 117 metabolites were identified by their MS/MS fragments. The results indicated that these four endophytic fungi are rich in amino acids and sugars, which are beneficial for the health of human beings. Also, in this study, by comparative metabolomics analysis, we found that the metabolic profile of pr10 is greatly different than those of other endophytic fungi (Figure 4), which is also demonstrated by the OPLS-DA analysis (Figure 4). This phenomenon showed that in the metabolite levels from the content to the diversity of metabolites, pr10 displays a more specific property than other endophytic fungi. Compared with those in pr6, contents of 31 metabolites are higher in pr10, such as D-arabitol that has been researched to found that it possesses antitumor activity by decreasing the survival rate of tumor cells. For pr7 vs pr10, in total, 47 metabolites were identified and filtered by their contents and found that these were synthesized by pr10 in a higher level (Figures 9 and 4). Also, there is an attractive metabolite, trehalose, and it has been reported that brartemin, a derivate of trehalose, has a good antitumor activity.
ability to inhibit the invasion of 26-L5 cells in colon cancer.\(^23\) The advantage is that trehalose could inhibit A549 tumor cells.\(^29\) In addition, the abundance of trehalose in pr10 explains the effective antitumor activity of pr10 on A549. Anything else, there are other 20 metabolites that were specifically synthesized at a high level in pr10. Among all of these 20 metabolites, except unknown compounds, carbohydrate compounds and amino acids and their derivatives are the main compounds. Also, it has been reported that amino acids such as phenylalanine and its derivatives, for example, L-phenylalanine compounds. Also, it has been reported that amino acids such as fucose, arabinoose, mannose, galactose, and glucose could act on McF-7 tumor cells of breast cancer, promote the proliferation of spleen cells, and stimulate the immune activity, thus inhibiting the growth of tumor cells.\(^24\) Moreover, in addition to direct antitumor activity, the metabolism of amino acids and sugar derivatives provides small molecular nutrients that are more readily absorbed by humans. The intake of these amino acids and carbohydrate-derived nutrients will also enhance human immunity, leading to an effective antitumor capacity.\(^30\) Due to the limitations of the current MS database, the unknown compounds, which were the main candidate metabolites in pr10, needed to be further studied in terms of metabolite identification. Moreover, such unknown metabolites have potential direct and indirect antitumor activities, which are helpful to make more rational use of endophytic fungi. Based on these above-mentioned advantages, it could be concluded that B. rapa L. with its specific endophytic fungi and metabolites synthesized by endophytic fungi could enhance the resistance of human beings in terms of tumors and other biotic and abiotic stresses.

In addition to the antitumor activity mentioned above, B. rapa L. has many other valuable characteristics such as antioxidant function and free-radical scavenging.\(^21\) It is the first time that comparative metabolomics was performed to systematically elucidate the antitumor mechanism of endophytic fungi of B. rapa L. The results manifested that B. rapa L. has unique kinds of endophytic fungi and such endophytic fungi could directly inhibit the tumor cells with their various biochemical metabolites. This research provides a theoretical basis and metabolic fingerprint database, at the biochemical metabolic level, for better use of B. rapa L. and its endophytic fungi to develop antitumor agents and give direction for digging out the potential value of antitumor medicinal plants at the biometabolic level in the future.

5. CONCLUSIONS

In view of the fact that previous experiments in vitro have proved that pr10 has antitumor properties there are no reports on the metabolomics of B. rapa L. endophytic fungi. Therefore, in this experiment, the method of comparative metabolomics was used to analyze the metabolomics of four strains of B. rapa L. endophytic fungi and the data were searched in the database. The metabolites of four strains of fungi were compared statistically, and the metabolism map of pr10 was drawn. The unique metabolites of endophytic fungus pr10 are rich in amino acids and sugar derivatives such as phenylalanine, D-arabitol, cellobiose, and trehalose. These metabolites have potential antitumor activity, especially trehalose whose antitumor activity on A549 has been reported.

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#### Notes

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### REFERENCES

1. Gond, S. K.; Mishra, A.; Sharma, V. K.; et al. Diversity and antimicrobial activity of endophytic fungi isolated from Nyctanthes arbor-tristis, a well-known medicinal plant of India. *MycoScience* 2012, 53, 113–121.

2. Petrini, Orlando. Fungal Endophytes of Tree Leaves. In *Microbial Ecology of Leaves*; Andrews, J. H.; Hirano, S. S., Eds.; Springer New York: New York, 1991; pp 179.

3. Ding, T.; Jiang, T.; Zhou, J. Evaluation of antimicrobial activity of endophytic fungi from Camptotheca acuminata (Nyssaceae). *Genet. Mol. Res. 2010*, 9, 2104–12.

4. Aly, A. H.; Debbab, A. D.; Kjer, J.; et al. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Diversity 2010*, 41, 1–16.

5. Refaei, J.; Jones, E.; Sakayaraj, J.; et al. Endophytic Fungi from Rafflesia cantleyi: species diversity and antimicrobial activity. *Mycoscience 2011*, 2, 429–447.

6. Rodriguez, R. J.; White, J. F., Jr; Arnold, A. E.; et al. Fungal endophytes: diversity and functional roles. *New Phytol. 2009*, 182, 314–30.

7. Stierle, A.; Strobel, G.; Stierle, D. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science 1993*, 260, 214–6.

8. Strobel, G.; Daisy, B. Bioprospecting for microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev. 2003*, 67, 491–502.

9. Yu, H.; Zhang, L.; Li, L. Recent developments and future prospects of antimicrobial metabolites produced by endophytes. *Microbiol. Res. 2010*, 165, 437–49.
(10) Woo, H. L.; Swenerton, K. D.; Hoskins, P. J. Taxol is active in platinum-resistant endometrial adenocarcinoma. Am. J. Clin. Oncol. 1996, 19, 290—1.

(11) Strobel, G.; Daisy, B.; Castillo, U.; et al. Natural products from endophytic microorganisms. J. Nat. Prod. 2004, 67, 257—68.

(12) Zou, W. X.; Tan, R. X. Recent advances on endophyte research. Acta Bot. Sin. 2001, 43, 881—892 in Chinese.

(13) Fernandes, F.; Valentao, P.; Sousa, C.; et al. Chemical and antioxidative assessment of dietary turnip (Brassica rapa var. rapa L.). Food Chem. 2007, 105, 1003—1010.

(14) Francisco, M.; Moreno, D. A.; Cartea, M. E.; et al. Simultaneous identification of glucosinolates and phenolic compounds in a representative collection of vegetable Brassica rapa. J. Chromatogr. A 2009, 1216, 6611—6619.

(15) Ferreres, F.; Valentao, P.; Pereira, J. A.; et al. HPLC-DAD-MS/MS-ESI screening of phenolic compounds in Pieris brassicae L. Reared on Brassica rapa var. rapa L. J. Agric. Food Chem. 2008, 56, 844—53.

(16) Ninomiya, M.; Efdi, M.; Inuzuka, T.; et al. Chalcone glycosides from aerial parts of Brassica rapa L. “hidabeni,” turnip. Phytochem. Lett. 2010, 3, 96—99.

(17) Hara, H.; Nakamura, Y.; Ninomiya, M.; et al. Inhibitory effects of chalcone glycosides isolated from Brassica rapa L. ‘hidabeni’ and their synthetic derivatives on LPS-induced NO production in microglia. Bioorg. Med. Chem. 2011, 19, 5559—68.

(18) Rafatullah, S.; Al-Yahya, M.; Mossa, J.; et al. Preliminary Phytochemical and Hepatoprotective Studies on Turnip Brassica rapa L. Int. J. Pharmacobiol. 2006, 2, 670—673.

(19) Jeong, J.; Park, H.; Hyeon, H.; et al. Effects of Glucosinolates from Turnip (Brassica rapa L.) Root on Bone Formation by Human Osteoblast-Like MG-63 Cells and in Normal Young Rats. Phytother. Res. 2015, 29, 902—909.

(20) Wang, S.; Tu, H.; Wan, J.; et al. Spatio-temporal distribution and natural variation of metabolites in citrus fruits. Food Chem. 2016, 199, 8—17.

(21) Hou, B. L. Studies on Antioxidation and Anti-tumor of Polysaccharides from Brassica rapa L. L Master Thesis, Xin Jiang Medical University, 2010.

(22) Christova, N.; Lang, S.; Wray, V.; et al. Production, Structural Elucidation, and In Vitro Antitumor Activity of Trehalose Lipid Biosurfactant from Nocardia farcinica Strain. J. Microbiol. Biotechnol. 2015, 25, 439—447.

(23) Igarashi, Y.; Mogi, T.; Yanase, S.; et al. Brartemicin, an inhibitor of tumor cell invasion from the actinomycete Nonomuraea sp. J. Nat. Prod. 2009, 72, 980—982.

(24) Chen, G.; Xu, J.; Miao, X.; et al. Characterization and antitumor activities of the water-soluble polysaccharide from Rhizoma Arisaematis. Carbohydr. Polym. 2012, 90, 67—72.

(25) Yen, C. T.; Wu, C. C.; Lee, J. C.; et al. Cytotoxic N-(fluorenyl-9-methoxycarbonyl) (Fmoc)-dipeptides: structure-activity relationships and synergistic studies. Eur. J. Med. Chem. 2010, 45, 2494—502.

(26) Kharwar, R.; Verma, V. C.; Kumar, A. Endophytic fungi: better players of biodiversity, stress tolerance, host protection and antimicrobial production. In A Text Book of Molecular Biotechnology; I K International Publishing House, 2009; pp 1033—1357.

(27) Daryoush, M.; Bahram, A. T.; Yousef, D. Protective effect of turnip root (Brassica rapa. L) ethanolic extract on early hepatic injury in alloxanized diabetic rats. Aust. J. Basic Appl. Sci. 2011, 5, 748—756.

(28) Khan, M. I. H.; Sohrab, M. H.; Rony, S. R.; et al. Cytotoxic and antibacterial naphthoquinones from an endophytic fungus, Cladosporium sp. Toxicol. Rep. 2016, 3, 861—865.

(29) Trehalose Liposomes Suppress the Growth of Tumors on Human Lung Carcinoma-bearing Mice by Induction of Apoptosis In Vivo. Anticancer Res. 2017, 37 6133 6139. DOI: 10.21873/ anticancerres.12062.

(30) Ohtsubo, Y.; Furukawa, M.; Fujinobu, Y.; et al. An immune adjuvant activity of mycolic acid-containing glycolipid, trehalose-2,3,6-trimycolate, derived from Gordona aurantiaca. Nihon Saikingu-ku Zasshi 1989, 44, 533—539.