Metabolic Changes of *Phomopsis longicolla* Fermentation and Its Effect on Antimicrobial Activity Against *Xanthomonas oryzae*

Choi, Jung Nam¹, Jiyoung Kim¹, Kannan Ponnusamy¹, Chaesung Lim¹, Jeong Gu Kim², Maria John Muthaiya¹, and Choong Hwan Lee¹*

¹Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Korea
²Genomics Division, National Academy of Agricultural Science (NAAS), Rural Development Administration (RDA), Suwon 441-857, Korea

Received: October 10, 2012 / Revised: October 24, 2012 / Accepted: October 26, 2012

Bacterial blight, an important and potentially destructive bacterial disease in rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), has recently developed resistance to the available antibiotics. In this study, mass spectrometry (MS)-based metabolite profiling and multivariate analysis were employed to investigate the correlation between time-dependent metabolite changes and antimicrobial activities against Xoo over the course of *Phomopsis longicolla S1B4* fermentation. Metabolites were clearly differentiated based on fermentation time into phase 1 (days 4–8) and phase 2 (days 10–20) in the principal component analysis (PCA) plot. The multivariate statistical analysis showed that the metabolites contributing significantly for phases 1 and 2 were deacetylphomoxanthone B, monodeacetylphomoxanthone B, fusaristatin A, and dicerandrols A, B, and C as identified by liquid chromatography-mass spectrometry (LC-MS), and dimethylglycine, isobutyric acid, pyruvic acid, ribofuranose, galactofuranose, fructose, arabinose, hexitol, myristic acid, and propylstearic acid were identified by gas chromatography-mass spectrometry (GC-MS)-based metabolite profiling. The most significantly different secondary metabolites, especially deacetylphomoxanthone B, monodeacetylphomoxanthone B, and dicerandrol A, B and C, were positively correlated with antibacterial activity against Xoo during fermentation.

**Key words:** Antimicrobial activity, metabolite profiling, *Phomopsis longicolla*, principal component analysis, *Xanthomonas oryzae*

*Xanthomonas oryzae* pv. *oryzae* (Xoo) is a major pathogen of rice causing bacterial leaf blight (BLB), which leads to serious economic losses to farmers [8, 9]. Recent reports indicate that the Xoo has produced resistance against various chemical substances, leading to a search of high effective disease preventive substance against Xoo [6]. Worldwide awareness about the usage of chemical substances against pest and disease pushes researchers to find alternative natural substances to control such plant microbes. Recent research proves that the *Phomopsis longicolla* fermentation products are found to be an effective antimicrobial compound. In our previous study, the antimicrobial activity of *Phomopsis longicolla* S1B4 secondary metabolites was examined against Xoo [6]. Identification of a novel phomoxanthone antibiotic made through metabolite profiling of *Phomopsis longicolla* in another study was reported (data not presented). However, the effect of fermentation on primary and secondary metabolites changes of *P. longicolla* S1B4 and its antimicrobial correlation against Xoo have not yet been reported.

Metabolomics is an emerging and rapidly developing science and technology that includes a comprehensive experimental analysis of metabolite profiles, either as targeted compounds or global metabolites [10]. This approach has been previously applied to the discovery of novel bioactive compounds and the optimization of the fermentation process [10, 11]. In metabolite profiling, it is preferable to use a wide-spectrum metabolite analysis technique that is rapid, reproducible, and stable during sample analysis [2]. Mass spectrometry (MS) is one of the techniques that can meet such demands. MS has been frequently used in earlier studies to detect and quantify the metabolome [12]. In the last few decades, a number of chemometric tools have been applied to the interpretation and quality assessment of MS-based metabolomic data.

In this study, the metabolic changes of *P. longicolla* S1B4 during fermentation and its antimicrobial activity against Xoo are reported. Principal component analysis
Fungal Strain and Fermentation Conditions

Metabolomics approaches. The results of this study may prove valuable with regard to the optimization of Phomopsis longicolla S1B4 fermentation for the production of antibacterial metabolites against Xoo using microbial metabolomics approaches.

MATERIALS AND METHODS

Fungal Strain and Fermentation Conditions

Phomopsis longicolla S1B4 was isolated from a plant sample collected in Hadong-gun Kyungnam Province, South Korea. Cultures maintained for 5 days at 25°C served as the seed culture. The mycelium was transferred into 200 ml of liquid medium and incubated under a shaker at 25°C for a period of 3 days and served as seed culture for the inoculation. This culture was inoculated again in fresh 200 ml liquid medium for a period of 20 days [7] at 25°C at 150 rpm and served as samples. From this, the samples collected at 2-day intervals underwent UPLC-Q-TOF-MS analysis to find out the metabolic changes and antimicrobial activity irrespective of the fermentation period.

Preparation of Endophytic Fungal Extracts

The mycelia were separated by filtering the broth followed by extracting with 100 ml of 80% aqueous acetone [3]. From the collected extracts, the solvent was removed by rotary evaporation followed by extracting twice with an equal volume of ethyl acetate (EtOAc). The EtOAc extract was dried and redissolved in methanol for analyzing the metabolic changes in UPLC-Q-TOF-MS. The remaining water layers of each sample after EtOAc extraction were concentrated and derivatized for the GC-MS analysis. Briefly, each water layer was lyophilized in a freeze dryer until completely dry, and then dissolved in 100 µl of pyridine (Sigma, St. Louis, MO, USA). To increase the volatility of polar compounds, acidic protons were then exchanged against the trimethylsilyl group using 50 µl of N-trimethylsilyl trifluoroacetamide (MSTFA; Sigma) at 70°C for 30 min.

Antimicrobial Activity

To detect the antimicrobial activity of the metabolites, the purified metabolites were tested against X. oryzae KACC 10331 by the agar diffusion method on solid medium and MIC analysis. Based on the results the correlation between antimicrobial activity and metabolite changes was examined. [6].

Secondary Metabolite Profiling by UPLC-ESI-Q-TOF-MS

UPLC performed on a Waters ACQUITY UPLC system (Waters) with a Waters ACQUITY BEH C18 column (100 × 2.1 mm i.d., 1.7 µm; Waters) was used for identification of significantly different secondary metabolites according to cultivation time in Phomopsis longicolla S1B4. The mobile phases consisted of A and B, high-purity water and acetonitrile, respectively, both containing 0.1% formic acid, and analysis was achieved at a flow rate of 0.3 ml/min with a 5 µl sample injection volume. The eluting conditions were optimized as follows: 0–1 min, 10% B; 1–3 min, linear gradient of B from 10% to 40%; 3–8 min, linear gradient of B from 40% to 70%; 8–10 min, linear gradient of B from 70% to 90%; 10–11 min, linear gradient of B from 90%–100%; 11–12.5 min, 100% B. The gradient was then switched to 10% B again and the column was equilibrated for 1.5 min before beginning the next injection. The column and autosampler were maintained at 30°C and 4°C, respectively.

For analyte detection, MS analysis was performed on a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) operating in negative-ion mode. The source temperature was set to 100°C, nebulization gas to 600 l/h at 200°C, and cone gas to 50 l/h. The capillary voltage and sample cone voltage were set to 3 kV and 30 V, respectively. The extraction cone voltages were set to 3.0 V. The mass spectrometer was used in V mode and the acquisition rate was set to 0.2 s, with a 0.02 s interscan delay. Leucine enkephalin was used as the lock mass (m/z 554.2615) at a concentration of 0.2 ng/µl and a flow rate of 3.0 µl/min. Argon was employed as the collision gas at a flow rate of 0.3 ml/min and the collision energy was set to 5 eV. Acquisition of m/z 100–1,500 was done with the applied dynamic range enhancement (DRF) function and centroided during acquisition.

Primary Metabolite Profiling by GC-EI-IT-MS

The remaining water layer from each fungal acetone extract was dried in a freeze dryer for 24 h. The lyophilized extracts were derivatized using 100 µl of pyrimidine (Sigma) and 50 µl of MSTFA at 70°C for 60 min [1]. GC-MS analysis was performed using a Varian 4000 MS system consisting of a CP-3800 gas chromatograph coupled to a 4000 ion-trap mass spectrometer equipped with a CP-8400 auto sampler (Varian). The samples were analyzed using a VF-1MS capillary column (30 m × 0.25 mm) coated with 0.25 µm low bleed polymer, a FactorFour column (Varian), and vaporized at 250°C in standard split mode (1:25). For analysis, the starting temperature was 70°C, which was held for 2 min, and then increased at a rate of 10°C/min to 300°C and held at this final temperature for 5 min. The flow rate of the helium carrier gas was adjusted to 1 ml/min. The interface and ion source temperatures were set at 200°C and electron impact ionization (70 eV) was utilized. The scan average was set to three microscans and full scanning with a range of 50–1,000 m/z. Metabolites were identified by comparing with the NIST 2005 database (version 2.0; FairCom Co., Columbia, MO, USA). All the samples were analyzed twice to decrease analytical error variation.

Data Processing

Secondary metabolite profiling data sets obtained by UPLC-Q-TOF-MS were preprocessed via peaking and alignment before multivariate statistical analysis, using a combination of software to maximize information regarding metabolite differences. Data analysis was performed by using Masslynx (version 4.1; Waters) to convert raw data into NetCDF file (*.cdf) format. Additionally, GC-EI-IT-MS data sets were converted into the NetCDF file format using Vx Capture (version 2.1; Adron Systems, LLC, Laporte, MN, USA) to perform baseline correction, alignment, and peak deconvolution with XCMS. The resulting metabolism sheets contain m/z retention time, and peak intensity (peak area) values for all samples were used for further analysis.
Multivariate Statistical Analysis

Multivariate statistical analysis comprising PCA and PLS-DA was performed using SIMCA-P+ software (version 12.0; Umetrics, Umeå, Sweden). PCA was run to obtain a general overview of the variance of metabolites, and PLS-DA was used to determine the metabolomic variation according to fermentation time. PCA models the main sources of variation in an unsupervised manner, whereas PLS-DA extracts as much of the class-separating variation as possible depending on fermentation time. Variable influence on the projection (VIP) values were calculated and used to identify the most significantly different metabolites for clustering of specific class separations. Univariate statistics for multiple classes were performed by breakdown and unpaired Student’s t-test using Statistica (version 7.0; StatSoft Inc., Tulsa, OK, USA). Box-and-whisker plots were generated in Statistica to show significant differences in chemistry measurements according to fermentation time. Pair-wise Pearson’s rank correlation coefficient (r) was calculated between antimicrobial activity against Xoo and important metabolites using the R software package (available at http://www.r-project.org/).

RESULTS AND DISCUSSION

Non-Targeted Metabolite Profiling of P. longicolla S1B4 Using Mass Spectrometry

For the identification of significant differences in metabolite composition according to fermentation time, endophytic fungal metabolites in 27 samples (three sets of incubated P. longicolla S1B4 mycelium that were collected for 20 days at 2-day intervals, excluding samples incubated for 2 days) were profiled using MS analysis combined with multivariate statistics. From the metabolite profile, 1,016 and 804 peaks were extracted by XCMS from UPLC-Q-TOF-MS and GC-EI-IT-MS data sets, respectively.

Owing to the qualitative similarity and complexity of the MS spectra from extracts of fermented P. longicolla S1B4, visualization of preliminary metabolite changes would be difficult; thus, chemometric multivariate statistical analysis was carried out by PCA [5]. Each normalized complete data set that evidenced changes in visible metabolite

![PCA and PLS-DA score plots derived from UPLC-ESI-Q-TOF-MS (A, C) and GC-EI-IT-MS (B, D) data sets for extracts of Phomopsis longicolla S1B4 collected for 20 days at 2-day intervals. These plots show that the metabolome could be clearly differentiated according to cultivation time into phase 1 (days 4–8) and phase 2 (days 10–20) by PC1, 54.6% (secondary metabolite profiling) and 41.4% (primary metabolite profiling).](image-url)
composition on score plots showed clear differences according to fermentation time (Fig. 1). The quality of the models was described by $R^2_X$ and $Q^2$ values. $R^2_X$ indicates the fitness and $Q^2$ indicates the predictability of models [4, 11]. The PCA score plots derived from UPLC-Q-TOF-MS data sets revealed that the secondary metabolite composition was clearly different depending on fermentation time and also explained 0.70 of the total variance ($R^2_X$) value (Fig. 1A). According to the PCA score plots derived from GC-EI-IT-MS data sets, the primary metabolite composition changed according to fermentation time and 0.63 of the $R^2_X$ (Fig. 1B) value could be explained. The predictability ($Q^2$) values of each data set were 0.67 and 0.58, respectively. As shown in these two PCA score plots, *P. longicolla S1B4* metabolites were clearly separated into two phases (phase 1: days 4, 6 and 8; phase 2: days 10, 12, 14, 16, 18, and 20) by PC1, 54.6% (secondary metabolite profiling by UPLC-Q-TOF-MS) and 41.4% (primary metabolite profiling by GC-EI-IT-MS) depending on fermentation, respectively.

**Multivariate Statistical Analysis of Non-Targeted Metabolite Profiling Data Sets**

In successive multivariate statistical analysis, individual supervised PLS-DA was carried out as described above to identify the significantly different metabolites that were changed according to fermentation time in *P. longicolla S1B4*. PLS-DA is commonly used to detect hidden variables that focus on class separation. Based on PLS-DA analysis, score plots derived from metabolite profiling data sets could generally be clustered depending on fermentation time, and a clear distinction between phases 1 and 2 was obtained (Fig. 1C and 1D). The first 12 PCs for secondary metabolite changes and 18 PCs for primary metabolite changes according to fermentation time accounted for 0.96 and 0.98 of the explained variance values, respectively. Thirteen secondary metabolites and 10 primary metabolites that changed significantly according to fermentation time were selected using variable importance in the projection (VIP > 1.0) and their $p$-value statistics ($p < 0.01$) (Table 1). Secondary metabolites were identified based on an in-house database and comparison with the literature [7, 13]. The most important primary metabolites contributing to the metabolite change according to fermentation time were identified using a NIST library and are summarized in Table 1. Metabolites such as dimethylglycine, isobutyric acid, pyruvic acid, ribofuranose, galactofuranose, fructose, arabinose, hexitol, myristic acid, and propylstearic acid were detected as significant variables according to fermentation time.

**Fermentation Time-Dependent Metabolite Changes in *P. longicolla S1B4***

In order to clearly interpret fermentation time-dependent metabolite changes, metabolite mass peak intensities were further analyzed using box-whisker plots. As displayed in Fig. 2A, the levels of 1–6 in secondary metabolites were significantly increased according to fermentation time up to 14 days. In a previous study, we reported an increase in antimicrobial activity against Xoo in *P. longicolla S1B4* with increasing fermentation time [6]. Therefore, fermentation time-dependent changes in these secondary metabolites can explain changes in *P. longicolla S1B4* antibacterial activity. Based on these combined results, the optimum fermentation time for maximal antibacterial activity and

<table>
<thead>
<tr>
<th>S.No</th>
<th>$^{a}$Rt</th>
<th>compound</th>
<th>S.No</th>
<th>$^{a}$Rt</th>
<th>compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.01</td>
<td>N. I.</td>
<td>14</td>
<td>5.21</td>
<td>Dimethylglycine</td>
</tr>
<tr>
<td>2</td>
<td>4.99</td>
<td>N. I.</td>
<td>15</td>
<td>7.7</td>
<td>Isobutyric acid</td>
</tr>
<tr>
<td>3</td>
<td>5.43</td>
<td>N. I.</td>
<td>16</td>
<td>8.2</td>
<td>Pyruvic acid</td>
</tr>
<tr>
<td>4</td>
<td>6.26</td>
<td>N. I.</td>
<td>17</td>
<td>14.72</td>
<td>Ribofuranose</td>
</tr>
<tr>
<td>5</td>
<td>6.93</td>
<td>N. I.</td>
<td>18</td>
<td>14.94</td>
<td>Galactofuranose</td>
</tr>
<tr>
<td>6</td>
<td>7.56</td>
<td>Dicerandrol A</td>
<td>19</td>
<td>15.97</td>
<td>Fructose</td>
</tr>
<tr>
<td>7</td>
<td>8.62</td>
<td>Deacetylphomoxanthone B</td>
<td>20</td>
<td>16.04</td>
<td>Arabinose</td>
</tr>
<tr>
<td>8</td>
<td>9.06</td>
<td>Dicerandrol B</td>
<td>21</td>
<td>17.18</td>
<td>Hexitol</td>
</tr>
<tr>
<td>9</td>
<td>9.37</td>
<td>Monodeacetylphomoxanthone B</td>
<td>22</td>
<td>20.77</td>
<td>Myristic acid</td>
</tr>
<tr>
<td>10</td>
<td>9.78</td>
<td>Dicerandrol C</td>
<td>23</td>
<td>23.6</td>
<td>Propylstearic acid</td>
</tr>
<tr>
<td>11</td>
<td>10.02</td>
<td>Fusaristatin A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>10.24</td>
<td>N. I.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>10.76</td>
<td>N. I.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$Rt, retention time. N.I., not identified.
Fig. 2. Metabolic changes of *Phomopsis longicolla* S1B4 during fermentation compared by box-whisker plots. The levels of six secondary metabolites (A) and ten primary metabolites (B) changed significantly during fermentation.
compound production in *P. longicolla* S1B4 appears to be 14 days after inoculation.

The changes in primary metabolites according to fermentation time are presented using box-whisker plots (Fig. 2B). The concentration of most of the primary metabolites increased significantly as the fermentation time increased up to 14 days; however, the levels of a few compounds including pyruvic acid and galactofuranose...
were found to be decreased. Pyruvic acid represents a key intersection in several metabolic pathways and can be made from glucose via glycolysis. It supplies energy to living cells in the citric acid cycle and can also be converted to carbohydrate, fatty acid, the amino acid alanine, and ethanol. Galactofuranose, the furanose form of galactose, has been used as a carbon source in fungi. Thus the observed decrease in pyruvic acid and galactofuranose concentrations with increased fermentation time reveals the continued growth of *P. longicolla* S1B4.

These results reveal that the concentration of metabolites during prolonged fermentation changed significantly, indicating that the optimal fermentation time for maximal production of biologically active secondary metabolites in *P. longicolla* S1B4 is 14 days after inoculation.

**Correlation Between Antimicrobial Activity and Metabolite Changes**

In order to clearly identify the relationships between antimicrobial activity and metabolites, a correlation heat map model was obtained using a data set of 23 metabolite variables and antimicrobial activity against Xoo (Fig. 3A). Consequently, most of the metabolites were positively correlated with antimicrobial activity against Xoo, except for pyruvic acid and galactofuranose. Among the metabolite-to-antimicrobial activity effect correlation pairs, 1–6 and metabolites No. 4, 5, and 13 (refer to Table 1 for metabolite numbers) exhibited strongly significant correlations (Pearson’s correlation coefficients $r > 0.95$, $p < 0.05$). Meanwhile, pyruvic acid and galactofuranose showed a negative correlation with antimicrobial activity (Pearson’s correlation coefficients $r > -0.80$, $p < 0.05$). In a previous report, we identified the antimicrobial activity of 3–5 against Xoo [6]. In addition, a highly positive correlation between seven metabolites and antimicrobial activity was observed in the metabolic correlation network (Fig. 3B).

This study demonstrates not only changes in metabolite composition in *P. longicolla* S1B4 as a function of fermentation time but also the effects of these changes on antimicrobial activity, using metabolite-to-activity correlation. UPLC-Q-TOF and GC-EI-mass spectrometry coupled with multivariate analyses were able to discern interesting and meaningful differences according to fermentation time. Furthermore, antimicrobial activity against Xoo appeared to be strongly and positively correlated with seven secondary metabolites. To the best of our knowledge, this is the first time that a metabolomics approach has been used to reveal compositional changes in metabolites according to fermentation time in *P. longicolla* S1B4. This study provides valuable information regarding the optimization of the fermentation process for bioactive compound production and describes an efficient way to screen for novel bioactive compounds from natural fungal sources.

**Acknowledgment**

This study was supported by National Research Foundation of Korea (NRF) grants funded by the Korea government (MEST 2010-0019306, MEST 2010-0027204), Republic of Korea.

**References**