Proteomics Based Study of Soybean and *Phakopsora pachyrhizi* Interaction

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**Abstract**

*Phakopsora pachyrhizi*, the causal agent of Asian soybean rust (ASR), has the potential to cause severe yield losses as all currently grown U.S. commercial soybean varieties are susceptible. In this proteomics study, we compared two soybean sibling lines, a resistant line 10G18 and a susceptible line 10G21 derived from Recombinant Inbred Line (RIL) population RN06-32-2 to understand the compatible and incompatible host-pathogen interactions at the molecular level. We compared the protein profile differences between the two lines over a time-course of 14 days with or without *P. pachyrhizi* inoculation using differential in-gel electrophoresis (DIGE). Approximately 70 differentially expressed spots between 10G18 and 10G21 lines with and without *P. pachyrhizi* inoculation were identified. Some of these spots, which were up- and down-regulated in resistant line 10G18, were sequenced using LC-MS/MS. Of the 70 differentially expressed protein spots, 31 up-regulated and 6 down-regulated spots in resistant line 10G18 were sequenced. These sequenced proteins were mostly involved in photosynthesis based on homology searches. The involvement in disease resistance for some of these differentially up-regulated proteins has been reported, indicating their possible role in soybean defense against ASR. However, further studies are necessary.

**Introduction**

Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* was first observed in Japan in 1902 and has since spread throughout the world (24). For several decades, the pathogen was slowly spreading in East Asia and Australia (10,30,31). ASR was first reported in Africa in late 1990s and in South America in 2001 (42). In November 2004, *P. pachyrhizi* reached North America. It was reported in Louisiana and several other southeastern states (33).

ASR is one of the most destructive foliar diseases of soybean (*Glycine max*) and has been known to drastically reduce the yield by causing severe defoliation (14). The threat of widespread infections of soybean during the growing season (July to November) has increased in the past few years since the pathogen, *P. pachyrhizi*, has a wide host range and is capable of overwintering on a number of alternative hosts including kudzu (*Pueraria lobata*) leading to high inoculum accumulation (24,26). The disease is mostly restricted to the Southern states due to the mild winter conditions, which are favorable for the ASR establishment (26,30). In general, the environmental conditions, such as temperatures ranging between 15-26°C, humidity as high as 80%, and good growth and full canopy development of the soybean crop, are most suitable to promote the development of ASR (19).

Currently, the control of ASR is mainly through fungicide applications. Cultural practices have also been recommended to reduce the impact of the pathogen on the crop (9). However, continuous increase in the use of fungicides
has led to several serious problems, such as fungicide resistance and toxicity to non-target aquatic organisms in the streams or ponds near the fields sprayed with the fungicides (23). Fungicides also have shown toxic effect to mammalian cells based on a laboratory assay (7). For these above reasons, improving host resistance of soybeans to ASR is considered the most viable alternative approach to fungicide applications.

As a long term control measure, efforts in screening for rust resistant soybean lines have identified six major R genes, \textit{Rpp1} to \textit{Rpp6} (13,20), that confer resistance to certain specific isolate(s) of \textit{P. pachyrhizi} (1,27,29). This is due to high genetic variation among different populations of \textit{P. pachyrhizi} (11,38). Therefore, developing durable genetic resistance against ASR has been difficult.

Recently, molecular based approaches have been used to understand the mechanisms of host-pathogen interactions and to identify the genes involved in host defense responses to ASR. Several microarray analyses of host responses to ASR have been reported (5,8,25,34,37) and many up-regulated genes associated with basic defense and down-regulated genes associated with metabolic pathways in the rust-infected susceptible soybean palisade and mesophyll cells have been identified. However, little information can be inferred from the microarray studies as to how these rust-induced genes respond at the protein level. Therefore, a proteomics-based investigation of host defenses is necessary to have a better understanding of how soybean responds to rust infection at the molecular level.

Proteomics is the study of the cellular proteome, defined as the set of proteins present in a biological unit (organism, organ, tissue, cell, or organelle) at a specific developmental stage and under determined external biotic and abiotic conditions (28). Use of proteomics offers several advantages such as understanding of post-transcriptional modifications and protein-protein interactions. The presence of a large number of unknown genes in the plant genome and the lack of correlation between mRNA and protein levels (16,41) can also be addressed by the use of proteomics. Recently, Lee et al. (2009) successfully examined the host-pathogen interaction between bean and \textit{Uromyces appendiculatus} using a proteomics approach. Similar studies have been done in soybean and \textit{Phakopsora pachyrhizi} (6) barrel-clover and \textit{Orobanche crenata} (3), wheat and \textit{Puccinia triticina} (32), rice and \textit{Magnaporthe grisea} (17), and maize and \textit{Aspergillus flavus} (4).

In recent years, a few proteomic studies have examined the soybean proteome in response to ASR (6,22,35,40) and proteome of ASR (21,35). Based upon these studies, many genes have been identified that are possibly involved in the defense response to ASR. Wang et al. (40) used resistant soybean genotype to identify the differentially expressed proteins whereas Park et al. (2013, unpublished) used two resistant and two susceptible soybean genotypes which are of different genetic background. In order to increase the chance of identifying true rust resistance associated proteins, we selected a pair of recombinant inbred lines that were segregating for rust resistance in the present study. We compared the proteome profile differences between the pair using a fluorescence based two-dimensional (2-D) differential in-gel electrophoresis (DIGE) technique to understand the host-pathogen interaction at the molecular level. It was found that proteins involved in photosynthesis and metabolism were generally down-regulated and the proteins involving in defense response were up-regulated upon rust infection.

**Materials and Methods**

**Plant material.** The resistant line 10G18 and susceptible line 10G21 derived from Recombinant Inbred Line (RIL) population RN06-32-2 were developed by Dr. David R. Walker (USDA-ASR, Urbana, IL). Their resistance and susceptibility to soybean rust spores collected in Louisiana were confirmed through repeated detached leaf and greenhouse inoculation studies (12). For proteomics comparisons, seeds of these soybean sibling lines were planted in 20-cm (8-inch) diameter plastic pots (4 seeds per pot) in the greenhouse. Plants were inoculated at R1 stage with rust spore suspension of $3 \times 10^4$ spores/ml and
control plants were mock inoculated with 0.01% Tween-20. Leaf tissues were collected at 0 h, 10 h, 1 day, 2 days, 3 days, 5 days, 8 days, 10 days, 12 days, and 14 days after inoculation (dai) and frozen immediately at -80°C until further use.

**Protein extraction.** The leaf tissue was ground in liquid nitrogen, and approximately 1 g was transferred to a 30-ml FEP Oak Ridge centrifuge tube and proteins were extracted using the phenol method (15). Protein pellets were washed twice with ice-cold 0.1 M ammonium acetate in 100% methanol containing 10 mM dithiotheritol (DTT) and washed twice with 80% acetone containing 10 mM DTT. During each washing step, pellet was completely re-suspended in solution by vortexing and precipitated by centrifugation for 10 min at 5000 × g and 4°C. Pellets were air-dried for 10 min and stored at -30°C until further use in electrophoresis.

**Two-dimensional gel electrophoresis, staining and analysis.** Protein pellets were solubilized in 130 µl of lysis buffer (2 M thiourea, 7 M urea, 4% w/v CHAPS, 30 mM Tris-HCl, pH 8.5) for 1 h at room temperature. The mixture was centrifuged for 10 min at 12,000 × g at 4°C. The supernatant was transferred to a new tube and the pH was adjusted to 8.5 using 50 mM NaOH. Protein concentration was then measured (Bradford, 1976) and adjusted to 6 µg/µl using lysis buffer. Ten µl (60 µg) of protein from inoculated or control samples were labeled with CyDye DIGE Fluor minimal dyes (Cy3 and Cy5, with dye swapping) according to the manufacturer’s instructions. In a separate tube, 60 µg of pooled proteins from all the samples in the experiment was labeled with Cy2 to be used as an internal control. The first and second dimension of gel electrophoresis was performed essentially as described (www.gelifesciences.com) in Ettan DALTtweleve large vertical system (GE Healthcare, Pittsburgh, PA). Analytical gels were scanned with Typhoon 9410 variable mode imager. Scanned gel images were analyzed in all possible combinations using Progenesis Samespots software (Nonlinear Dynamics, www.nonlinear.com) to identify differentially up- or down-regulated protein spots between inoculated and control leaf samples from compatible and incompatible interactions.

**Mass spectrometry and protein identification.** Preparative gels, which contained a minimum of 500 µg proteins, were stained with 0.125% Coomassie Brilliant Blue G-250, and scanned. Protein spots in preparative gels corresponding to those that showed differential expression in analytical gels were selected for spot picking and peptide sequence analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for protein identification. The mass spectra were searched using the MASCOT search engine (www.matrixscience.com) using the following search parameters: Viridiplantae (green plants), trypsin, up to one missed cleavage, carbamidomethylation of cysteine and oxidation of methionine, peptide tolerance 1.2 Da, mass value MH+ and monoisotopic.

**Comparison of Protein Profile Differences between Sister Lines under Non-infection Conditions**

Protein profiles in soybean leaves between 10G18 (resistant) and 10G21 (susceptible) 10 h after mock inoculation (hai) were compared using Progenesis Samespots software to identify differentially expressed proteins (Fig. 1). The spots that were significantly up-regulated (P < 0.05) in line 10G18 are shown in the Figure 1. These six spots (spot IDs: 0004, 0777, 0376, 0613, 0705, 0748) were also up-regulated in line 10G18 at time points other than 10 h (Fig. 2, Table 1).
Fig. 1. Differential in-gel electrophoresis (Dige) of protein profile differences between soybean sibling lines 10G18 (resistant) (A) and 10G21 (susceptible) (B) 10 h after mock inoculation. Protein spot IDs were indicated with red and black arrows for up-regulated and down-regulated proteins, respectively.
Fig. 2. Representative gel sub-sections of the selected differentially expressed protein spots between soybean sibling lines 10G18 (resistant) control and 10G21 (susceptible) control. ID numbers on top of the gels refer to the spot ID presented in Tables 1 and 2.
Table 1. The identification of spots up-regulated in resistant line (10G18) in comparison to the susceptible line (10G21).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spot ID</th>
<th>Time Point</th>
<th>Control Folds Up-regulation</th>
<th>Infected Folds Up-regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0748</td>
<td>5 d, 10 h</td>
<td>4.5</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>0705</td>
<td>2 d, 14 d</td>
<td>6.5</td>
<td>2.6</td>
</tr>
<tr>
<td>3</td>
<td>0777</td>
<td>10 h, 2 d</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>0376</td>
<td>0 h, 10 h</td>
<td>2.2</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>0613</td>
<td>0 h, 10 h</td>
<td>4.1</td>
<td>3.0</td>
</tr>
<tr>
<td>6</td>
<td>004</td>
<td>2 d, 10 h</td>
<td>2.0</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* Ratio of average normalized spot volume across five time points in line 10G18 compared to line 10G21.

Comparison of Protein Profile Differences between Sibling Lines under Infection Conditions

In a similar way, protein profiles were compared between 10G18 (resistant) and 10G21 (susceptible) 10 h after *P. pachyrhizi* inoculation (hai). The six spots that were up-regulated in 10G18 at 10 hai after mock inoculation were also significantly up-regulated (*P* < 0.05) 10 h after *P. pachyrhizi* inoculation and are shown in the Figure 3. Gel sub-sections of these up-regulated spots are shown in Figure 4 and Table 1. Some of these differentially up-regulated proteins might be involved in the early defense response of soybean against *P. pachyrhizi* infection. However, further studies are necessary.

MS/MS Analysis of Differentially Expressed Proteins

These six proteins were recovered from preparative gels and sequenced using LC-MS/MS (Table 2). Spot 0376 showed high sequence similarity to the deduced amino acid sequence of ribulose bisphosphate carboxylase large chain (YP_538747) from soybean. Spot 0613 was identified as probable chloroplastic-like fructose-bisphosphate aldolase 2 (XP_003537836). Peptide sequences from spot 0705 were identified as ribulose bisphosphate carboxylase small chain 1 precursor (P00865). Spot 0748, spot 0777 and spot 0004 were identified as uncharacterized protein (NP_001235654), serine hydroxymethyl transferase 5 (NP_001237509) and stem 28 kDa glycoprotein (NP_001238459), respectively. Overall, three of the identified up-regulated proteins are involved in photosynthesis and four are unknown proteins.

Significance

Identification of the host proteins involved in defense response to *P. pachyrhizi* is the first step in enhancing resistance in soybeans against rust. This can be achieved by the use of RIL derived sibling lines that share high genetic similarities. This can increase our chance of identifying important host proteins involved in resistance mechanism against rust. In addition, study of differentially expressed proteins between resistant and susceptible soybean sister lines can lead to identification of proteins/genes that are directly involved in resistance to soybean rust. Up-regulation of photosynthesis related proteins in resistant line during rust infection may help soybean plants to better defend against rust infection. Similar results, such as increases in oxygen evolving enhancer protein 1 (OEE1) and thylakoid luminal protein involved in photosynthesis upon pathogen infection were reported in earlier studies (36,39).

Future studies will focus on confirming the importance of these differentially expressed proteins in soybean resistance to rust infection. Once their involvement in soybean resistance to rust is verified, these genes can be used to improve resistance to *P. pachyrhizi* infection by either genetic engineering of soybean or through the conventional breeding approach to transfer the useful genes into target soybean germplasm.
Table 2. Protein identifications and properties of spots differentially expressed in resistant line (10G18) and susceptible line (10G21) in response to *Phakopsora pachyrhizi* inoculation.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spot ID</th>
<th>Putative protein</th>
<th>NCBI accession number</th>
<th>Time point</th>
<th>Fold change&lt;sup&gt;u&lt;/sup&gt;</th>
<th>No. of matching peptides&lt;sup&gt;v&lt;/sup&gt;</th>
<th>Peptide Sequence&lt;sup&gt;w&lt;/sup&gt;</th>
<th>Mascot score&lt;sup&gt;x&lt;/sup&gt;</th>
<th>% Sequence coverage&lt;sup&gt;y&lt;/sup&gt;</th>
<th>pI&lt;sup&gt;z&lt;/sup&gt;</th>
<th>MW (Da)&lt;sup&gt;z&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>1</td>
<td>0748</td>
<td>Uncharacterized protein LOC100499761</td>
<td>NP_001235654</td>
<td>All the time points</td>
<td>1.2</td>
<td>3</td>
<td>gkDIVELIaagrlatvpsgggaVaaPgggaAAAAPaaeaklatvPsgggaavavaaPggGaaaAPaaeakk</td>
<td>132</td>
<td>39</td>
<td>4.36</td>
<td>11439</td>
</tr>
<tr>
<td>2</td>
<td>0705</td>
<td>Ribulose bisphosphate carboxylase small chain 1, chloroplastic, precursor</td>
<td>P00865</td>
<td>5 days</td>
<td>1.1</td>
<td>2</td>
<td>IIIFDNVRtaYPNGfIR</td>
<td>68</td>
<td>9</td>
<td>8.87</td>
<td>20060</td>
</tr>
<tr>
<td>3</td>
<td>0748, 0777, 0004</td>
<td>Uncharacterized protein LOC100499761</td>
<td>NP_001235654</td>
<td>All the time points</td>
<td>1.2</td>
<td>3</td>
<td>gkDIVELIaagrlatvpsgggaVaaPgggaAAAAPaaeaklatvPsgggaavavaaPggGaaaAPaaeakk</td>
<td>132</td>
<td>39</td>
<td>4.36</td>
<td>11439</td>
</tr>
<tr>
<td>4</td>
<td>0613</td>
<td>Probable fructose-bisphosphate aldolase 2, chloroplastic-like, predicted</td>
<td>XP_003537836</td>
<td>12 days</td>
<td>1.3</td>
<td>9</td>
<td>eAAWGLaR aAQDALLFR aGSYADEIVk saAYyqQGar aSPqTVADYTLK lasIGLENTENR tvVSIPNGPSsLAVK dKASpQTVAHYTLK rlasiGENTENR</td>
<td>580</td>
<td>20</td>
<td>8.24</td>
<td>42925</td>
</tr>
</tbody>
</table>

<sup>t</sup> Spot identification number (Figs. 1 and 3).
<sup>u</sup> Ratios represent fold change relative to susceptible line (10G21).
<sup>v</sup> Number of identified unique peptides by Mascot MS/MS ion search.
<sup>w</sup> lower case letters indicate no confidence based on Mascot MS/MS ion search.
<sup>x</sup> Obtained from Mascot.
<sup>y</sup> The values indicate the percentage of sequence coverage of identified peptides.
<sup>z</sup> Theoretical values.
Fig. 3. Differential in-gel electrophoresis (DIGE) of protein profile differences between soybean sibling lines 10G18 (resistant) (A) and 10G21 (susceptible) (B) 10 h after inoculation with *P. pachyrhizi*. Protein spot IDs were indicated with red and black arrows for up-regulated and down-regulated proteins, respectively. ID numbers on top of the gels refer to the spot ID presented in Tables 1 and 2.
Fig. 4. Representative gel sub-sections of selected differentially expressed protein spots between soybean sibling lines 10G18 (resistant) infected and 10G21 (susceptible) infected. ID numbers on top of the gels refer to the spot ID presented in Tables 1 and 2.
Fig. 5. (A) MS/MS fragmentation of peptide TNAENEFVTIK (Spot 0376), (B) Mascot interpretation (Mascot is a web based tool for predicting the protein peptide sequence based on the differences of mass over charge ratio of two adjacent ions).
Literature Cited


