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Study of Host-Fungus Interactions between Soybean and Phakopsora Pachyrhizi Using Proteomics

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STUDY OF HOST-FUNGUS INTERACTIONS BETWEEN SOYBEAN AND *PHAKOPSORA PACHYRHIZI* USING PROTEOMICS

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LIST OF ABBREVIATIONS

2-D DIGE	2-dimensional difference gel electrophoresis
2-DE	2-dimensional gel electrophoresis
ABC	ATP-binding cassette
ASR	Asian soybean rust
CBB	Coomassie brilliant blue
CER	Carbon exchange rate
CHAPS	3-([3-Cholamidopropyl]-dimethyl-ammonio)-1-propane-sulfonate)
CHI	Chalcone Isomerase
DTT	Dithiothreitol
EFR	Elongation factor EF-Tu
ESI	Electrospray ionization
ESTs	Expressed sequence tags
ET	Ethylene
FLS2	Flagellin receptor
FNR	Ferredoxin-NADP reductase
HR	Hypersensitive reaction
IAA	Iodoacetamide
IEF	Iso-electric focus
IPG	Immobilized pH gradient
JA	Jasmonic acid
LRR	Leucine-rich repeat
MALDI	Matrix-assisted laser desorption/ionization
MAMP	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MS/MS	Tandem mass spectrometry
NBS	Nucleotide-binding site
NPR1	Nonexpressor of pathogenesis-related genes 1
OEE1	Oxygen evolving enhancer protein1
ORFs	Open reading frames

OsPR10	Rice pathogenesis-related protein class 10
PAMP	Pathogen-associated molecular patterns
PBZ1	Probenazole-inducible protein
PEG	Polyethylene glycol
pI	Iso-electric point
PMF	Peptide mass fingerprinting
POX	Peroxidase
PR protein	Pathogenesis-related protein
PRRs	Pattern recognition receptors
PSII	Photosystem II
R gene	Resistance gene
RB	Reddish-brown
RbohD	Membrane-localized NADPH oxidases
RLKs	Receptor-like protein kinase
ROS	Reactive oxygen species
RT-PCR	Real-time PCR
SA	Salicylic acid
SAGE	Serial analysis of gene expression
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
STAND	Signal transduction ATPases with numerous domains
TCA	Trichloracetic acid
TEMED	N,N,N^9,N^9, -tetramethyl-ethylenediamine
TIR	TOLL/INTERLEUKIN -1 RECEPTOR
TLP	Thaumatin-like protein

ABSTRACT

Asian soybean rust, caused by *Phakopsora pachyrhizi*, is an emerging disease in the continental U. S. and resistant commercial varieties have not been reported. In an effort to understand the interactions during rust infection of soybean, protein profile changes were examined over a 14-day period in soybean leaves of one susceptible commercial line (Pioneer 93M60) with or without soybean rust inoculation using proteomics in this study. Forty protein spots differentially expressed after rust inoculation were identified and fourteen of them were recovered and sequenced. These included proteins involved in plant defense, stress, metabolism, and other biological processes. During the time-course of rust infection, several proteins were significantly induced as early as 10 hai, such as pathogenesis-related protein 10 (PR10) and cytosolic glutamine synthetase. PR10 and chalcone isomerase 1 (CHI1), putative plant defense proteins, were further examined using quantitative real-time PCR (qRT-PCR). *CHI1* transcript, the most abundant among three *CHIs*, was highly induced by soybean rust infection at 10 hai. Transcript level of *PR10* was also significantly induced at 10 hai, 6 and 8 dai. We found two accessions (PI417089A and PI567104B) showed consistent immune response to a Louisiana soybean rust isolate using both detached leaf assay and greenhouse inoculation after screening of 12 accessions. Fungal biomass, determined using qRT-PCR, increased significantly at 2 days after infection in susceptible lines, whereas no or little increase was detected in the resistant lines. Protein profiles of these two resistant and two susceptible lines (PI548631 and 93M60) were compared to find proteins involved in host resistance at the molecular level. Eight and 15 proteins were identified as up-regulated spots at 1 day after rust infection in both resistant accessions after comparing to the susceptible lines, PI548631 and 93M60, separately. Sixteen spots were sequenced, and they belonged to plant defense, signaling, and photosynthesis. We

found that most up-regulated protein spots were identified as potential plant defense-related proteins in this study using proteomics and proteomics approach may be an effective means to identify novel proteins potentially involved in host resistance.

CHAPTER 1
INTRODUCTION

1.1Justification

Asian soybean rust (ASR), caused by an obligate parasite *Phakopsora pachyrhizi*, was first reported in Japan in 1902 and then in China in 1940. The pathogen spread to Africa in 1996 South America in 2001 (Yorinori et al. 2005), and finally to the continental United States in 2004 (Schneider et al. 2005). In the U.S., soybean rust was found in nine states in 2005, and it was reported in 16 states in 2009 (http://sbr.ipmpipe.org/). Further, recent studies indicated that *P. pachyrhizi* could survive the mild winter conditions in the southern U.S., and therefore, the pathogen poses a continuous threat to soybean production in the U.S. (Jurick II et al. 2008; Park et al. 2008). *P. pachyrhizi* is a very aggressive foliar pathogen of soybean and causes yield losses up to 80% (Hartman et al. 2001; Yorinori et al. 2005). Currently, all U.S. commercial soybean cultivars are susceptible to the fungus, and the only method to control this disease is timely and costly application of fungicides. Hence, there is an urgent need to develop varieties that are resistant or tolerant to ASR to reduce its potential to cause yield losses in the U.S. In an effort to develop resistance to ASR, germplasm screening studies were conducted. Soybean accessions resistant to *P. pachyrhizi* isolates collected from different countries, such as India, Taiwan, Nigeria, Paraguay, Vietnam, and the U.S. were identified (Miles et al. 2008; Pham et al. 2009; Twizeyimana et al. 2007). Four single dominant genes, *Rpp1*, *Rpp2*, *Rpp3*, and *Rpp4*, have been reported that confer resistance to specific isolates of *P. pachyrhizi* (Bromfield and Hartwig 1980; Hartwig 1986; Hidayat and Somaatmadja 1977). However, it has been reported that the effectiveness of resistance can be overcome by virulent ASR isolates collected from other places (Hartman et al. 2005). Due to this reason, developing genetic resistance has not been successful. Recently, molecular based approaches have been conducted to find genes involved in host

defense, as well as to study how soybean rust infects the host and how the host responds to pathogen attack at the molecular level. Microarray studies have been conducted to identify genes involved in host resistance after soybean rust infection using resistant lines containing *Rpp1* or *Rpp2* resistance genes (Choi et al. 2008; van de Mortel et al. 2007). However, microarray is limited to analysis of gene expression at the transcript level, which usually has poor correlation with expression at the protein level. Proteomics is the study of proteins which have vital function in all celluar mechanisms, and this approach is very useful for studying proteins differentially expressed between different treatments and proteins undergoing post-translational functional modifications. A proteomic approach has been successfully used to examine host-pathogen interactions in previous studies between bean and *Uromyces appendiculatus* (Lee et al. 2009), barrel-clover and *Orobanche crenata* (Castillejo et al. 2009), wheat and *Puccinia triticina* (Rampitsch et al. 2006), rice and *Magnaporthe grisea* (Kim et al. 2004), and maize and *Aspergillus flavus* (Chen et al. 2004).

1.2 Objectives

1) Identify host and fungal proteins induced during compatible interaction using proteomics
2) Screen soybean accessions to find resistant accessions to Louisiana isolate
3) Study differentially expressed proteins between resistant and susceptible accessions with and without fungal infection using proteomics
4) Characterize these proteins to understand host-fungus interactions
5) Verify the importance of promising host proteins in disease resistance using a virus induced gene silencing.

In this study, the long term goal is to enhance host resistance to *Phakopsora pachyrhizi* infection and control soybean rust disease through understanding host-parasite interactions using a proteomic approach.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Asian Soybean Rust

2.1.1 Asian Soybean Rust History

Asian soybean rust, caused by *Phakopsora pachyrhizi*, obligate biotrophic plant fungus, was first discovered in Japan in 1902 and then in China in 1940. The pathogen spread to Africa in 1996 South America in 2001 (Yorinori et al. 2005), and finally to the continental United States in 2004 for the first time (Schneider et al. 2005). In the U.S., nine states reported soybean rust in 2005, 16 states in 2006, 19 states in 2007, and 16 states in 2008 and 2009 (http://www.usda.gov/soybean rust/). The disease poses a serious threat to the soybean industry in the U. S.

2.1.2 Disease Symptoms and Yield Loss

The three most common host reactions to infection by *P. pachyrhizi* that have been described are tan reaction, reddish-brown (RB) reaction, and immune reaction. Tan is a susceptible reaction characterized by tan lesions with many uredinia and prolific sporulation. RB is a resistant reaction classified by reddish-brown lesions with few uredinia and little to moderate sporulation, and immune is another resistant reaction with no visible lesions or uredinia (Bromfield *et al*. 1984; Bromfield and Hartwig 1980). Dark reddish brown lesions with few uredinia and extensive necrosis indicate a semi-compatible interaction, and tan lesions with two or more uredinia without extensive necrosis indicate a compatible interaction (Sinclair and Hartman 1999). Lesions are angular and 2-5 mm in diameter. They are often restricted by leaf veins but may also appear on petioles, stems, and pods (Miles et al. 2007). The disease symptom generally appears in the lower canopy and proceeds upward. Sporulating lesions are most easily identified on the lower leaf surface. As the disease progresses, high lesion densities result in

premature leaflet drop and early maturation (Sinclair and Hartman 1999). Color of a lesion varies depending on its age and the interaction between the soybean genotype and the race of pathogen. In 1966, physiological races of *P. pachyrhizi* were first reported when a set of nine single urediniospore isolates were inoculated onto six soybean and five legume accessions (Lin 1966). Six pathotypes were found based on their reactions on the legume accessions, while the reactions of the nine isolates were similar on all six of the soybean genotypes. Different reaction types of *P. pachyrhizi* on soybean accessions were first reported in Australia (McLean and Byth 1980). They found that one isolate was virulent on the cultivar "William" but avirulent on the accession PI200492, while another isolate was virulent on both soybean genotypes. Other studies have reported significant variation in virulence among isolates collected from different geographical areas (Pham et al. 2009; Oloka et al. 2008).

Heavily infected plants result in reduced seed weight and fewer pods and seeds. Significant losses have been reported in Thailand (10-40%), India (10-90%), southern China (10-50%), Taiwan (23-90%), Japan (40%), and Brazil (30-75%) (Hartman et al. 1991; Hartman et al. 1999; Miles et al. 2007).

2.1.3 Soybean Rust Host Range

Host range of *P. pachyrhizi* is very broad. It infects over 95 species of legumes including soybean, related *Glycine* species (Rytter et al. 1984), and kudzu (*Pueraria lobata*), which is an invasive fast-growing vine spread wide in the southern U.S. Yellow sweet clover *(Melilotus officinalis*), vetch (*Vicia dasycarpa*), medic (*Medicago arborea*), lupine *(Lupinus hirsutus*), green and kidney bean (*Phaseolus vulgaris*), lima and butter bean *(Phaseolus lunatus*), and cowpea or blackeyed pea (*Vigna unguiculata*) are common hosts for the fungus as well.

2.1.4 Asian Soybean Rust Life Cycle

The Asian soybean rust life cycle begins 1 to 2 h after inoculation (hai) with urediniospore germination and germ-tube formation when incubated in a dark, humid condition and at a conductive temperature (Bonde et al. 1976). Appressoria forms from the tips of germ tubes along anticlinal walls of epidermal cells within 2 hai (Bonde et al. 1976; Koch et al. 1983). High humidity for about 6 hrs is required for successful infection. Penetration pegs form by 7 hai, and hyphae directly enter epidermal cell. The penetrated epidermal cell loses cellular organization within 24 hai and collapses by 4 days after inoculation (dai) (Koch et al. 1983). The primary hyphae grow between spongy mesophyll cells and occasionally form hausteria between the plant cell wall and plasma membrane where the fungus obtains nutrients and secretes effector proteins (Hahn et al. 1997; Staples 2001; Voegele and Mendgen 2003) between 1 dai and 2 dai. When effector proteins are not recognized by the the host plant, the fungus proceeds to further colonize the intercellular spaces of the spongy mesophyll by producing secondary hyphae and additional haustoria. Urediniospores are produced at 7 to 9 dai (Marchetti *et al*. 1975). Urediniospores, which are the means of disease spread, are released by rupture of the epidermis at 9 dai, and uredinia can disseminate spores up to 4 weeks (Koch et al. 1983) (Fig. 2.1).

2.1.5 Environmental Conditions

Temperature is one of the key factors affecting the rust life cycle. Many studies have been conducted to identify favorable temperature conditions for disease development. Natural infection by *P. pachyrhizi* is favored by a maximum temperature of 26 °C to 29 °C and a minimum temperature of 15 °C to 17 °C (Levy 2005). No infection was observed at temperatures 29.5°C or higher (Marchetti et al. 1976; Melching et al. 1989). Rust disease development also was greatly inhibited when mean night temperature drops below 14 °C, and was stopped when night temperature reaches below 9 °C (Tschanz et al. 1986; Melching et al. 1989).

When temperatures were kept at 4 to 5 °C or below, *P. pachyrhizi*, urediniospores lost their viability in 5 days (Patil et al. 1997). However, at 9 °C, viability lasted up to 27 days (Tan 1994). Kochman (1979) reported significantly reduced germination when dry spores were exposed to temperatures of 28.5-42.5 °C. According to a disease assessment study, climatic conditions of soybean growing regions in the U.S. are suitable for soybean rust epidemics (Pivonia and Yang 2004). Recent studies indicated that *P. pachyrhizi* could survive the mild and short winter conditions in the southern U. S., and the disease therefore poses a continuous threat to soybean production in the U. S. (Jurick II et al. 2008; Park et al. 2008). Kudzu also has been found as a host, on which soybean rust urediniospores can survive the winter in the southern U.S. states, such as Alabama, Florida, Georgia, Louisiana, Mississippi, and Texas (Pivonia and Yang 2005).

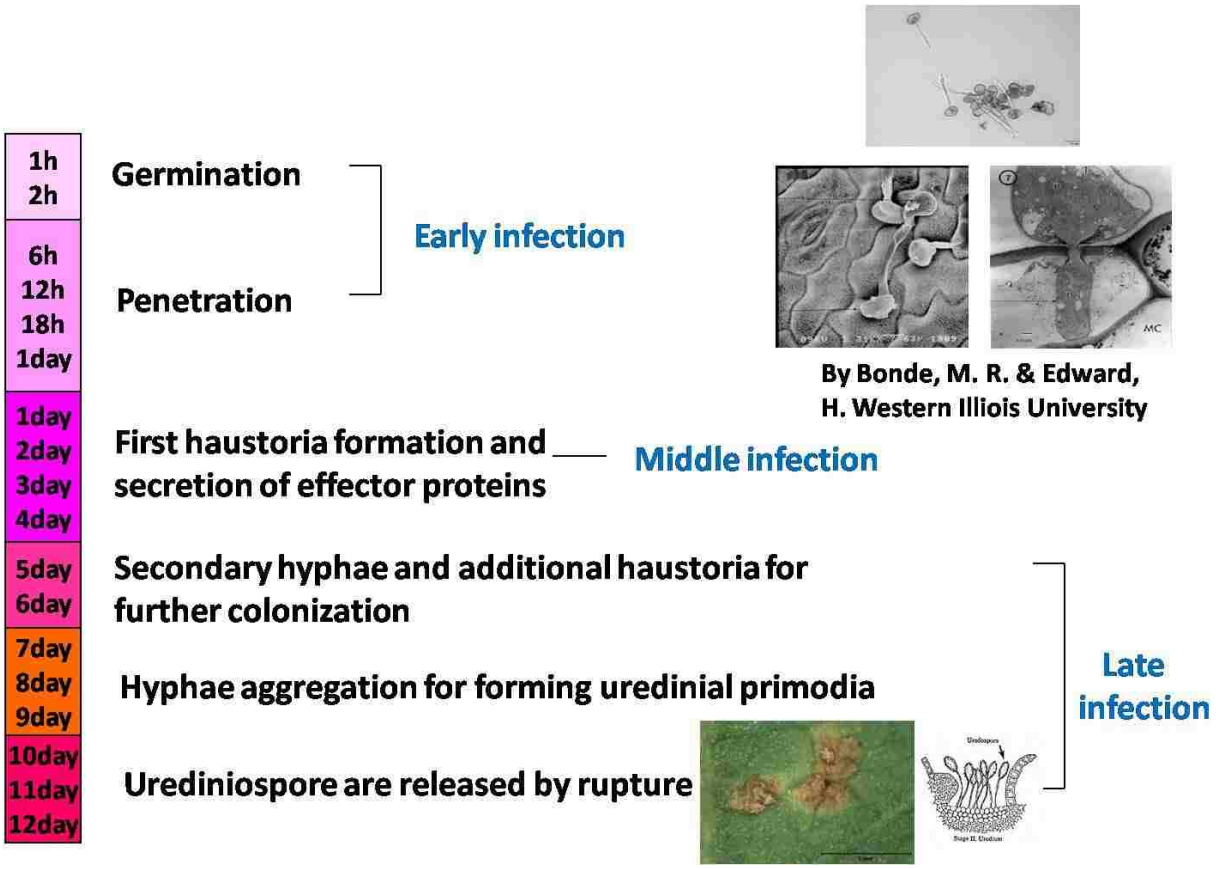


Figure 2.1. Asian soybean rust (*Phakopsora pachyrhizi*) life cycle.

2.1.6 Control Methods

Most of the studies addressing rust disease control methods focus on developing host resistance and the use of fungicides. Some limited cultural practice and biological control research also are being conducted (Desborough 1984). The first host plant resistance to *P. pachyrhizi* was reported in Taiwan in the 1960s from the field screening of soybean accessions (Lin 1966). Soybean lines with specific single-gene resistance to *P. pachyrhizi*, such as *Rpp1*, *Rpp2*, *Rpp3*, *Rpp4* and *Rpp5* were identified (*Rpp1*, Hartwig and Bromfield, 1983; *Rpp2*, Hidayat and Somaatmadja, 1977; *Rpp3*, Bromfield and Hartwig, 1980; *Rpp4*, Hartwig 1986; *Rpp5*, Garcia et al. 2008). However, these lines showed resistance to limited rust isolates and became ineffective soon after they were found (Hartman et al. 2005). Accessions containing *Rpp1* showed an immune reaction to a few soybean rust isolates, including India 73-1, while soybean plants containing *Rpp1* and the other genes resulted in RB reaction with no or sparsely sporulating uredinia after inoculation of most rust isolates (Bond et al. 2006). An example of ineffective single gene resistance can be found in the soybean accession PI230970 containing *Rpp2*. It was identified as resistant in field evaluations in 1971-1973, but a few susceptible lesions were found on the plant in 1976. By 1978, most of the lesions were the susceptible tan reaction type (Bromfield 1984).

Due to the ineffective single gene resistance and lack of information on mechanisms of host resistance to soybean rust, soybean growers are dependent on costly fungicide applications. There are only a few fungicide compounds currently registered for foliar application on soybean in the U.S. These include chloronitrile (Bravo and Echo), Strobilurins (Quadris, Headline), Triazoles (Topguard, Folicur) and strobilurin and triazole premixes (Quadris Xtra). Many studies also focused on developing effective application methods for delivering enough fungicide, uniformly, into the lower portion of the soybean canopy after flowering (Miles et al. 2007). The

economics of fungicide application in regard to timing, number of sprays, and which fungicides to use for the most cost effective control also is studied (Christiano and Scherm 2007).

2.2 Proteomics

2.2.1 Comparison of Genomic and Proteomic Approaches

In recent years, many advanced molecular techniques have been used to identify genes and their functions. Gene expression at the transcriptional level has been studied using differential screening methods, such as differential display, real time PCR (RT-PCR), suppressive subtractive hybridization, serial analysis of gene expression (SAGE), and DNA microarray. DNA microarray has been used extensively to study differential gene expressions and regulations in response to pathogens/stresses, as well as to identify known or unknown defense genes.

However, the limitations of these mRNA based techniques also are clear. First, mRNA expression levels do not necessarily correlate with protein levels because of large differences in mRNA stability and protein turnover (Gygi et al. 1999). Second, mRNA analysis gives us little information as to whether a particular transcript is being translated into a protein, and whether the encoded protein is active or has a function because many proteins involved in plant defense mechanisms have activity and function at a particular subcellular location only after they undergo post-translational modifications, such as removal of signal peptides, phosphorylation or glycosylation (Zivy and de Vienne 2000), which cannot be revealed through mRNA analysis. Third, mRNA cannot be used for studying profile changes of secreted proteins (Kim and Kang 2008). Fourth, mRNA analysis will not predict how many protein species will be produced from one gene through alternative splicing or post-translational modifications. It has been reported that a number of protein species can be translated from a single gene as a result of alternative splicing during the resistance response. Several mechanisms of alternative splicing have been

reported for plant resistance (R) genes belonging to the Toll/interleukin -1 receptor (TIR)-nucleotide-binding site (NBS)-leucine-rich repeat (LRR) class. Alternative transcripts of R genes have premature stop codons that produce truncated open reading frames (ORFs) resulting TIR-NBS or TIR-NBS with first several LRRs (Jordan et al. 2002; Zhang and Gassmann 2007). Fifth, mRNA analysis will give limited information on proteins which have function only when they form complexes with other proteins or RNA molecules. Moreover, transcriptomics or functional genomics approaches to study soybean are not effective because soybean has genome duplications and a long generation time (Komatsu and Ashan 2009). In these cases, the proteomic approach would be a powerful tool to compensate for the drawbacks of transcriptomics for analyzing the functions of the plant genes or proteins.

Proteomics is the study of function and structure of proteins which have vital roles in various physiological metabolic pathways in a cell. Proteomics has wide applications: 1) identification of all the proteins that make up a proteome, 2) studying the structure and function of the complete set of proteins produced by the genome of an organism, including post-translational modification and glycosylation, 3) studying protein interaction with small or large molecules, and 4) studying expression pattern of proteins by a time-course under certain physiological conditions (Bradshaw 2008). Moreover, proteomics is a promising tool for analyzing the gene responses of non-model plants, especially those whose genome has not been completely sequenced (Komatsu and Ahsan 2009). In addition, proteomics can identify the missing proteins that have not been identified due to alternative splicing or uncharacterized proteins that were not revealed by genome analysis (Bradshaw 2008). Therefore, proteomics is considered a complementary approach to genomics or transcriptomics because the first step of the proteomics is matching proteins to genes already known through database analysis.

2.2.2 Principle of 2-Dimensional Electrophoresis

Two-dimensional gel electrophoresis (2-DE), which separates proteins based on their pI (isoelectric point) in the first dimension and their molecular weight in the second dimension, has been a powerful technique in the 35 years since its first report (O'Farrell 1975). Its application was accelerated with the development of immobilized pH gradient (IPGs) and protein identification through mass spectrometry (MS). The major steps of 2-DE are sample preparation and protein solubilization, protein separation by 2-DE, protein detection, analysis of protein pattern using software, peptide sequencing using MS, and protein identification through homology analysis.

2.2.2.1 Development of Protein Extraction Methods for Better Resolution and High Reproducibility

To achieve a high resolution of 2-DE, protein samples are denatured, disaggregated, reduced and solubilized to completely disrupt the molecular interactions and to maximize the chance that each spot represents an individual protein. There is no single method for sample preparation that can be used universally on all kinds of plant tissues. It should be optimized for the different types of tissues. Many research groups have developed protein extraction methods that are optimized for protein extraction and reproducibility using different plants (rice, soybean, tomato) or different tissues (leaf, root, stem, flower, or fruit) (Carpentier et al. 2005; Saravanan and Rose 2004). Recently, different extraction methods were compared for different organs of soybean, such as hypocotyls, root, seed, and leaf (Aghaei et al. 2009; Sarma et al. 2008; Xu et al. 2006). They demonstrated that a phenol-based method or/TCA-acetone-phenol based method is more effective than TCA/acetone precipitation. Especially, the modified phenol-based methanol/ammonium acetate precipitation method, which is originally developed by Hurkman and Tanaka (1986), resulted in the best resolution of soybean leaf proteome. It is believed phenol

and acetone or ethanol precipitation can efficiently remove interfering compounds, such as proteolytic enzymes, salts, lipids, nucleic acids, polysaccharides, plant phenols and/ or highly abundant proteins (Sarma et al. 2008). This method also provides better results for recalcitrant plant tissues compared to TCA/acetone precitation method. Along with optimization of protein extraction methods, development of a pre-fractionation method also is important to remove house-keeping and highly abundant proteins, such as Rubisco in plants and albumin in animal systems and to allow detection of low abundance proteins, such as membrane protein or transcription factors. These methods include: polyethylene glycol (PEG) fractionation followed by Mg/NP-40 extraction buffer (Kim et al. 2001), sequential extraction with a series of reagents based on differential protein solubility (Santoni et al. 2000), subcellular proteome for compartments, including chloroplasts, mitochondria, nuclei, and the extracellular matrix (Chivasa et al. 2005), and secreted proteome (Oh et al. 2005).

High reproducibility and high resolution are achieved by the immobilized pH gradient (IPG) strip using the bifunctional immobiline reagents, a series of 10 chemically well idefined acrylamide derivatives. They form a series of buffers with different pK values between pK1 and 13 and copolymerize with the acrylamide matrix. It produces a very stable pH gradient allowing true steady-state iso-electric focusing (IEF) with increased reproducibility (Blomberg et al. 1995).

2.2.2.2 Various Staining Methods for Protein Spot Visualization

After protein samples are separated on the SDS-polyacrylamide gel by pI and MW, protein spots must be visualized by universal or by specific staining methods. Important properties of staining methods are low cost, high sensitivity (low detection limit), high linear dynamic range for quantitative accuracy, reproducibility, and compatibility with post-electrophoretic protein identification methods such as MS. However, there is no single staining method that satisfies all these requirements (Görg et al. 1998). Universal protein detection

methods include coomassie brilliant blue (CBB), silver, and SYPRO Ruby staining. CBB staining method is widely used because of low price, simplicity, reproducibility and compatibility with peptide sequence methods like MS. However, low abundance proteins cannot be detected by CBB because of its low sensitivity (the detection limit is about 100-500 ng per spot). Thus, just a few hundred spots can be detected even if a large amount of protein is loaded (Candiano et al. 2004). Silver staining method is more sensitive than CBB because its detection limit is as low as 0.1 ng protein per spot. But it is more expensive and complicated. Also, it lacks reproducibility (Syrovy and Hodny 1991) and is incompatible with subsequent protein analysis like MS due to protein cross-linkage (Yan et al. 2000). Recently, acidic silver staining method using zincon and sodium thiosulfate as silver ion sensitizers, which is compatible with MS, has been developed (Jin et al. 2008). A fluorescent staining method, SYPRO Ruby, has a detection limit of 0.25–1ng per band. This method is simpler and more sensitive than the silver staining method, with better linear dynamic range, reproducibility, and MS compatibility; however, the cost of stain is higher, and special handling and instruments are required for data acquisition of fluorescent-stained gels (Berggren et al. 2000). Recently, 2-D fluorescence difference gel electrophoresis (2-D DIGE) technique employing two to three different fluorescent dyes for control and treated samples was used in differential proteomics to reduce the time-consuming, laborious, and gel-to-gel variation of multistep 2-DE. DIGE method was first developed by Ünlü et al. in 1997. Two samples are labeled *in vitro* using two different fluorescent cyanine minimal dyes differing in their excitation and emission wavelengths, then the two labeled samples are mixed and separated in the same gel. After consecutive excitation with two different wavelengths, the images are overlaid and normalized, where protein differences (up- or down-regulated, modified proteins) between two samples are visualized, which can significantly reduce the time required to identify differentially expressed proteins. However, the cost of dyes is prohibitive for

daily use. Other fluorescent detection methods include Pro-Q Diamond and Pro-Q Emerald, which also are compatible with MS. They are used for detecting phosphorylated protein (detection limit is 1-2 ng) and glycosylated protein (detection limit is 5-20 ng), respectively.

2.2.2.3 Gel Image Analysis and Spot Indentification

Gel images are subjected to computer-based analysis using 2-D software for spot detection, quantification and matching. Differentially expressed unknown protein spots can be identified using tandem mass spectrometry (MS/MS) technique for high throughput protein identification. There are two methods to characterize proteins. First is a 'top-down' strategy, in which intact whole proteins are ionized by electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), and then introduced into mass analyzer. Second is a 'bottom-up' strategy, in which proteins are digested into smaller peptides using proteases, such as trypsin or pepsin, and the collected peptides are then introduced into a mass analyzer (Schad et al. 2005). Characteristic pattern of peptides can be used for identification using peptide mass fingerprinting (PMF). *De novo* peptide sequences also can be obtained for protein identification if the peptide of interest is further analyzed through a second MS (Jorrín-Novo et al. 2009).

2.2.3 Various Applications of Proteomics and Proteomic Studies on Host-Parasite Interactions

Proteomics has be applied in many different studies such as characterizing the biochemistry of organelles (Ashan and Komatsu 2009; Carroll et al. 2008), protein differential expression induced by a specific genotype (i.e. wild, transgenic, mutant) (Herman et al. 2003; Kang et al. 2007), a developmental stage (i. e. germination) (Kim et al. 2009), fruit development and ripening (Giribaldi et al. 2007), leaf senescence (Hebeler et al. 2008), programmed cell death (Kim et al. 2008a), effect of hormone (auxin, giberellins, abscisic and jasmonic acid) (He et al. 2008; Kim et al. 2008b, and external conditions (i. e. symbioses, biotic stress; pathogen or insect,

abiotic stress; drought, temperature, UV light, heavy metal, and oxidative stress) (Patterson et al. 2007; Sharma et al. 2008; van Noorden et al. 2007; Wan and Liu 2008; Wang et al. 2009; Xu et al. 2008; Zhu et al. 2007). It has also been used to identify proteins that have undergone posttranslational modification (Nuhse et al. 2007), and to study protein interaction (Popescu et al. 2007). Many studies on host-parasite interaction using proteomics have been conducted in plant species with complete genome sequence information, such as rice (Kim et al. 2004, 2008a), *Arabidopsis* (Chivasa et al. 2006), and maize (Campo et al. 2004; Chen et al. 2007). Proteomic approach also has been extensively used for studying host-parasite interactions in other plant systems whose complete genomic sequences are not available yet, such as wheat (Rampitsch et al. 2006), tomato, barley, and peach (Chan et al. 2007; Geddes et al. 2008; Houterman et al. 2007). A few legume species have also been studied using a proteomic approach, such as *Lotus japonicus* (Wienkoop and Saalbach 2003), *Medicago truncatula* (Colditz et al. 2004), and *Pisum sativum* (Curto et al. 2006), to gain a better understanding of the molecular basis of host-parasite interactions.

Proteins differentially expressed due to pathogen infection have been identified in many model plant systems. When rice was infected by the rice blast fungus, *Magnaporthe grisea*, a number of proteins (two RLKs, glucanase 1 and 2, POX22.3, PBZ1, and OsPR10) were found differentially expressed in response to the infection. Western blot analysis showed that induction of TLP, OsRLK, PBZ1 and OsPR10 was faster and higher in the incompatible interactions than in compatible ones. They also studied localization of two PR10 family members, PBZ1 and OsPR10, and found that PBZ1 localized in the mesophyll cells under the attachment sites of appresoria, whereas OsPR10 was present in the vascular tissues. They concluded that temporal and spatial differences contributed to the different host defense against the pathogen (Kim et al. 2004). Differentially expressed proteins were identified in *Arabidopsis* cell culture after

Fusarium fungal elicitor treatment. Elicitor responsive proteins included molecular chaperones, oxidative stress defense proteins, mitochondrial proteins, and enzymes of diverse number of metabolic pathways, such as peroxidase (PR-9), glutathione *S*-transferase, and fructose-bisphosphate aldolase (Chivasa et al. 2005). In maize responding to *Fusarium verticillioides* infection, the following proteins were induced in embryo: β-1,3-glucanases, glutathione *S*-transferase, glyceraldehydes 3-phosphate dehydrogenase, and fructose-bisphosphate aldolase (Campo et al. 2004). The majority of *M. truncatula* root proteins produced in response to *Aphanomyces euteiches* infection are in the PR10 family, whereas others were putative cell wall proteins and enzymes of the phenylpropanoid-isoflavonoid pathway (Colditz et al. 2004). Interestingly, many hosts induce similar proteins in response to pathogens. A number of PR proteins (PR1, PR5, etc), POX and SOD were differentially accumulated in response to pathogen attack in two pea lines that were susceptible or resistant to powdery mildew, *Erysiphe pisi* (Curto et al. 2006). Seven host and 22 fungal proteins were found consistently upregulated in a susceptible interaction between wheat and *Puccinia triticina* (leaf rust) (Rampitsch et al. 2006). This study also demonstrated that a proteomics approach can be used to identify not only the host proteins, but also fungal proteins.

2.2.4 Proteomic Study on Soybean

A few studies of soybean interactions with symbionts, such as *Bradyrhizobium japonicum*, using proteomics have been reported (Hempel et al. 2009; Wan et al. 2005). Recently, several studies reported differential expression of soybean proteins in response to various stresses, including toxic metals (Sobkowiak and Deckert 2006), salinity (Aghaei et al. 2009), flooding (Shi et al. 2008), and UV-B (Xu et al. 2008). The proteomic approach has also been used to compare allergens between cultivars and wild-type soybean (Joseph et al. 2006) and to analyze expression of allergens in transgenic soybean (Herman et al. 2003). Protein profiles of

soybean leaf, root hair, and during seed filling also have been examined (Brechenmacher et al. 2009; Hajduch et al. 2005; Xu et al. 2006). The completion of the soybean genome sequence (Schmutz et al. 2010) will accelerate the functional genomics in soybean, including proteomics.

2.3 Plant Defense Mechanism

2.3.1 Microbe/Pathogen-Associated Molecular Patterns-Triggered Host Resistance

Plants don't have mobile defense cells or a somatic adaptive immune system like mammals, so they rely on the innate immune system and systemic signals starting from infected cells, where each individual plant cell can autonomously initiate a defense mechanism (Chisholm et al. 2006). There are two layers in the plant defense system. First layer is a basal defense mechanism which is based on membrane-anchored pattern recognition receptors (PRRs) (Panstruga et al. 2006). Plant PRRs consist of leucine-rich reapeat (LRR) receptor-like kinases, such as flagellin receptor FLS2, its coreceptor BRI1 (brassinosteroid insensitive 1)-associated kinase 1 (BAK1), and the receptor for bacterial elongation factor EF-Tu called EFR (Nürnberger and Kemmerling 2006). Membrane-bound proteins containing peptidoglycan-binding LysM domain is also a PRR, which binds the fungal MAMP, chitin (Wan et al. 2008). PRRs monitor conserved micro/pathogen-associated molecular patterns (MAMP or PAMP), such as lipopolysaccharides and flagellin, and bind to them directly or associate with MAMP binding proteins. Recognition of MAMP by PRRs induces intracellular signal transduction through mitogen-activated protein kinase (MAPK) cascades (van Ooijen et al. 2007). This event induces plant–specific transcriptional factors like WRKY to activate defense genes (Nicaise et al. 2009).

Early MAMP-triggered responses include secretion of defense-related proteins, such as PR-1 for immune response by vesicle-associated and SNARE protein-mediated focal secretion through exocytosis pathway (Kwon et al. 2008), translocation of toxic chemical derived from the non-toxic precursor in cytosol to the extracellular space for antimicrobial activity through ATP-

binding cassette (ABC) transporter family, apoplast acidification, and extracellular generation of reactive oxygen species (O_2^- and H_2O_2) (ROS) by membrane-localized NADPH oxidases (RbohD). Especially, ROS triggers the hydroperoxidation of membrane phospholipids producing toxic mixtures of lipid hydroperoxides, resulting in disruption of cell membranes (Agrios 1997). In contrast, proteins in polyglucan callose or papilla biosynthesis to produce structural barriers in the extracellular space showed a relatively late response (Panstruga et al. 2006). Although these events induce weak immune responses, it is effective to stop colonization of most disease-causing microbes (Mehta et al. 2008; Takken and Tameling 2009).

The first layer, basal defense mechanism, can be overcome by the secreted extracellular enzymes of pathogen, such as catalases (pectin esterases, polygalaturonases, xylanases, pecto lyases and cellulases), superoxide dismutase (SOD) protecting microbes from oxidative stress through inactivating O_2^- and H_2O_2, and effector proteins (Mehta et al. 2008; Takken and Tameling 2009). Microbial effector proteins, such as AvrPto, AvrPtoB, and HooM1, break the basal innate immune system by reducing the level of MAMP signaling or by targeting the secretory defense mechanism (Boller 2008). The second layer of host defense is effective against specific pathogens that can successfully break through the first layer and is based on highly polymorphic resistance (R) proteins from host (Takken and Tameling 2009).

2.3.2 Resistance (R) Protein-Triggered Host Resistance

During infection, pathogens produce effector proteins, which suppress the first layer of host defense mechanisms (basal defense) and clear the way for infection through the intercepting of signaling induced by innate immune response or by targeting the secretory defense machinery (Panstruga et al. 2009). But some effectors are recognized by host R proteins, consequently causing the host to develop strong defense responses. In contrast to PRRs, R proteins specifically recognize avirulence proteins (effector) that are not conserved between species or even isolates

of a given pathogen (Jones and Dangl 2006). Due to the one-to-one relationship between a plant R gene and matching *avirulence* (*Avr*) gene in a pathogen, this type of immunity is called gene-for-gene resistance (Flor et al. 1942). Plant R proteins (RPM1, RPS2, PRF, N, RPS4, MLA and L6) are present in the cytosol and interact either directly or indirectly with Avr proteins, such as AvrRpm1, AvrB, and AvrRpt2 like PRRs-mediated resistance (Marathe and Dinesh-Kumar 2003). Common structure modules of plant R proteins contain a central nucleotide binding (NB) domain, a LRRs in the C-terminal and a coiled-coil (CC) or TOLL/Interleukin-1 receptor (TIR) domain at the N-terminal (van Ooijen et al. 2007). The NB domain is part of the NB-ARC domain. Proteins containing NB-ARC domain belong to the family of STAND (signal transduction ATPases with numerous domains) NTPase and may regulate signal transduction (Takken and Tameling 2009). In this model, the ADP-bound state represents the "OFF" state and ATP-bound state is the "ON" state of the protein. Interaction with effector proteins induces a conformational change that enables ADP, which is tightly bound to the R protein in the absence of effector, to be exchanged for ATP. With ATP binding, R proteins can activate host defenses through an unknown pathway (Tameling et al. 2006). Many R proteins need cytosolic chaperones HSP90 and HSC70, as well as co-chaperones RAR1 and SGT1 for their function (Azevedo et al. 2002). This folding module is typically necessary in the synthesis of autorepressed receptors (Liu et al. 2004). This autoinhibition mechanism enables R protein expression to be inhibited in the absence of the pathogen but rapidly activated upon attack. Otherwise, constitutive R protein expression can cause host cell death.

R protein mediated resistance is achieved by accumulation of reactive oxygen species (ROS) that lead to hypersensitive reaction (HR) at the site of infection and activation of defense genes encoding pathogenesis-related (PR) proteins or antimicrobial compound. This differs both quantitatively and kinetically from PRRs-mediated resistance (Dangl and Jones 2001). Previous

studies showed that similar defense-related gene expression changes were found in compatible and incompatible interactions between *A. thaliana* and the fungus *Peronospora parasitica* (Maleck et al. 2000), and oxidative burst occurs not only in the incompatible interaction, but also in the compatible interaction (Goodman and Novacky 1994). They concluded that resistance may be the result of a faster response and at a higher quantity of defense-related gene expression in the incompatible interaction than the compatible interaction, in which susceptible plants failed to stop pathogen growth due to a slow response of and/or low levels of defense-related gene expressions. Since the output from both PRRs and R protein-triggered resistance is similar, it is possible that they share the same signaling pathway. Recently, it was reported that several intracellular R proteins are translocated into the nucleus and involved in direct transcriptional reprogramming of host cells for rapid immune response (Panstruga et al. 2006).

In addition to HR, systemic acquired resistance (SAR) is also induced in uninfected tissue by R protein-mediated resistance reaction (Sticher et al. 1997). Unlike HR causing localized cell death only at the infection site, SAR provides a long-lasting resistance throughout the whole plant to stop infections by a broad range of pathogens (Lamb and Dixon, 1997). Initiation of SAR is related to the elevated levels of phenolic hormone salicylic acid (SA). Activated intracellular R proteins stimulate SA biosynthesis and signaling through the nucleo-cytoplasmic regulators, EDS1 and PAD4 (Bartsch et al. 2006). Induced SA interacts with oligomeric NPR1 (nonexpressor of pathogenesis-related genes 1) to reduce it to monomeric NPR1 because only monomeric NPR1 can be translocated from cytoplasm to nucleus where it modulates the expression of antimicrobial and secretory pathway genes needed for SAR (Mou et al. 2003). Other phytohormones, gaseous ethylene (ET) and jasmonic acid (JA) also regulate SAR. It has been proposed that cross-talking between different hormone systems enable host plants to respond appropriately to a particular mode of pathogen infection and to integrate biotic and

abiotic stress stimuli. The JA and ET pathways are integrated at the transcriptional machinery by closely related induction of defense pathway. In addition, SA and ET/JA signaling pathways are mutually antagonistic (Kunkel and Brooks 2002).

CHAPTER 3
IDENTIFICATION AND CHARACTERIZATION OF PROTEINS INDUCED OR SUPPRESSED DURING A SUSCEPTIBLE HOST-PATHOGEN INTERACTION BETWEEN SOYBEAN AND *PHAKOPSORA PACHYRHIZI*

3.1 Introduction

Asian soybean rust, caused by an obligate parasite *Phakopsora pachyrhizi* Sydow, was first reported in Japan in 1902 and later in China in 1940. The pathogen spread to Africa in 1996 South America in 2001 (Yorinori et al. 2005), and finally to the continental United States in 2004 (Schneider et al. 2005). In the U.S., soybean rust was found in nine states in 2005, and it was reported in 16 states in 2009 (http://sbr.ipmpipe.org/). *P. pachyrhizi* is a very aggressive foliar pathogen of soybean and causes yield losses up to 80% (Hartman et al. 2001; Yorinori et al. 2005). Currently, all U. S. commercial soybean cultivars are susceptible to soybean rust disease, which can only be controlled through timely fungicide applications. Further, recent studies indicated that *P. pachyrhizi* could survive the mild winter conditions in the southern U.S. and poses a continuous threat to soybean production in the U.S. (Jurick II et al. 2008; Park et al. 2008). The development of resistant soybean varieties is a high priority for soybean breeders as it represents a more effective, economical and sustainable long-term control measure for producers when compared to expensive fungicide applications.

As part of a concerted effort to develop resistant cultivars, soybean germplasm collections have been screened in the past years and soybean lines with single-gene resistance, including *Rpp1, Rpp2, Rpp3, Rpp4,* and *Rpp5* have been identified (*Rpp1*, Hartwig and Bromfield, 1983; *Rpp2*, Hidayat and Somaatmadja, 1977; *Rpp3*, Bromfield and Hartwig, 1980; *Rpp4*, Hartwig 1986; *Rpp5*, Garcia et al. 2008). However, these lines showed resistance to limited rust isolates and became ineffective soon after they were found (Hartman et al. 2005).

Another approach to develop resistant cultivars is to understand host-pathogen interactions at the molecular level and use this knowledge for marker-assisted selection. Recent microarray studies showed that most of the differentially expressed host genes in a susceptible interaction between soybean and *P. pachyrhizi* were defense-related genes and stress-inducible genes (Panthee et al. 2007), and the majority of up-regulated genes in soybean containing *Rpp1* or *Rpp2* resistance genes also were defense-related (Choi et al. 2008; van de Mortel et al. 2007). Both resistant and susceptible soybean lines were found to induce a similar set of genes after rust infection, but the induction at transcript level was observed one day earlier in the resistant line than in the susceptible one (van de Mortel et al. 2007). It was suggested that this temporal difference in gene expression may be key in the successful infection of soybean. However, whether or not the up-regulation of these genes has been translated into an increased production of their corresponding proteins, expressing biological functions during the host-parasite interactions has yet to be examined.

Proteomics, a complementary approach to genomics, has been effectively used to identify host and fungal proteins involved in host pathogen interactions in wheat and *Puccinia triticina* (Rampitsch et al. 2006), rice and *Magnaporthe grisea* (Kim et al. 2004), and *Arabidopsis* and *Alternaria brassicicola* (Oh et al. 2005). The present study used a proteomic approach to examine the protein profile differences between Asian soybean rust-inoculated soybean leaves and non-inoculated leaves to identify proteins that were differentially expressed in rust inoculated leaves compared to control leaves. Protein profiles also were compared during the time course of rust infection to determine how early the host responds to pathogen attack and how protein levels are changed at different infection stages. Two of the infection-induced proteins, chalcone isomerase 1 and pathogenesis-related protein 10 (PR10), were further investigated to determine their roles in soybean defense against rust infection.

3.2 Materials and Methods

3.2.1 Chemicals

All chemicals used in this study were of analytical grade. Isoliquiritigenin (2', 4', 4', 6'-tetrahydroxychalcone), KCN, β-methoxyethanol, Dowex (100-200 mesh), ethylenediaminetetra acetic acid (EDTA), and iodoacetamide (IAA) were purchased from Sigma (St. Louis, MO). Phenol was purchased from EMD Chemicals Inc. (Gibbstown, NJ). Immobilized pH gradient (IPG) buffer (pH 3.0 to 10.0 NL and pH 4.0 to 7.0), Immobiline Dry Strip (pH 3.0 to 10.0 NL, 18 cm and 24 cm and pH 4.0 to 7.0, 18cm), dithiothreitol (DTT), and bromophenol blue were purchased from GE Healthcare Biosciences (Pittsburgh, PA). Tris, urea, Dodeca Siver Stain Kit, sodium dodecyl sulfate (SDS), Coomassie brilliant blue R-250, 3-([3-Cholamidopropyl]-dimethyl-ammonio)-1-propane-sulfonate) (CHAPS), ammonium persulfate, and N,N,N^9,N^9, -tetramethyl-ethylenediamine (TEMED) were purchased from Bio-Rad (Hercules, CA). Tween 20, β-mercaptoethanol, and sucrose were purchased from AMRESCO Inc. (Solon, OH). Ammonium acetate, acetone, and glacial acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). Methanol, glycerol, sodium phosphate, and potassium phosphate were purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ).

3.2.2 Soybean Plant Inoculation

Soybean rust (*Phakopsora pachyrhizi*) urediniospores were collected from naturally infected soybean leaves (10/20/2007)) at the Ben Hur Experiment Station, Louisiana State University Agricultural Center, Baton Rouge. Spores were stored at -80°C until use. Soybean cultivar 93M60 (Pioneer, Johnston, IA) was grown in 20-cm-diameter plastic pots (4 seeds per pot) in the greenhouse. After 3 weeks, 24 plants (at R1 to R2 growth stage) were inoculated by spraying 100 ml of a urediniospore suspension (1×10^5 urediniospores per ml) in sterile water containing 0.01% Tween 20. Plants were maintained at 25°C with 100% humidity in the dark for

2 days before being moved back to greenhouse. Control (non-inoculated) plants were sprayed with 100 ml of sterile, distilled water containing 0.01% Tween 20 and were treated in the same manner. The fourth to sixth trifoliate leaves from non-inoculated and inoculated plants were harvested at 14 days after inoculation and frozen immediately with liquid nitrogen. For the time-course experiment, inoculated and non-inoculated leaves were collected 10 h, 1, 2, 3, 4, 6, 8, 10, 12, and 14 days after inoculation (dai). They were stored at -80°C until extraction. This experiment was conducted independently three times.

3.2.3 Protein Extraction

Soybean leaves (1 g) were ground in liquid nitrogen using a mortar and pestle. Protein was extracted using phenol followed by methanolic ammonium acetate precipitation (Hurkman and Tanaka, 1986). Briefly, the ground leaf powder was transferred to a 30 ml oak ridge tube (Nalgene, Rochester, NY), and 2.5 ml of 0.1 M Tris-HCl (pH 8.8) saturated phenol and 2.5 ml of extraction buffer (0.1 M Tris-HCl, pH 8.8, 10 mM EDTA, 0.4% β-mercaptoethanol, 0.9 M sucrose) were added to the tube and homogenized for 1 min using a Polytron PT 3100 homogenizer (Kinematica Inc., Newark, NJ) at 10,000 rpm. Mixtures were agitated for 30 min at 4°C followed by centrifugation for 10 min at 5000 g and 4°C. Phenol layer (top layer) was transferred to a new tube and another 2.5 ml of Tris-HCl (pH 8.8) saturated phenol was added to the original tube to repeat the extraction one more time. This second phenol phase was transferred to a new tube. An equal volume of protein extraction buffer was added to the combined phenol phase, and the mixture was agitated for 30 min at 4°C followed by centrifugation for 10 min at 5000 g and 4°C. Phenol phase was transferred to a new tube and the extracted proteins were precipitated by adding 5 to 10 volumes of 0.1 M ammonium acetate in 100 % methanol (pre-chilled at -80°C) at -80°C for at least 2 hours. A protein pellet obtained by centrifugation at 4000 g for 30 min at 4°C was washed twice with ice-cold 0.1 M ammonium

acetate in 100% methanol containing 10 mM DTT and washed twice with 80% acetone containing 10 mM DTT. During each wash step, the pellet was recovered by centrifugation at 5000 g for 10 min at 4°C. The pellet was dried in a fume hood for 10 min and stored at -30°C until use. Protein extraction for each leaf sample was conducted twice.

3.2.4 First-dimension Gel Electrophoresis

Pellets were solubilized in lysis buffer (8 M urea, 4% CHAPS, 40 mM DTT, and 2% wt/vol IPG buffer) (Görg et al. 1998). The mixture was centrifuged for 10 min at 14,000 rpm at 20°C. Supernatant was transferred to a new tube and protein concentration was measured (Bradford, 1976). Immobiline DryStrips of pH 3 to10 NL or pH 4 to 7 were re-hydrated overnight in 350 µl of rehydration solution (8 M urea, 2% CHAPS, 20 mM DTT, bromophenol, and 0.5% IPG buffer) with 150 µg of protein for silver staining (18 cm strip, analytical gel) and in 450 µl of rehydration solution with 700 µg protein for Coomassie blue staining (24 cm strip, preparative gel). Isoelectric focusing (IEF) was performed at 20°C for a total of 7 h for 18 cm and 10 h for 24 cm strips under the following conditions: 1 h at 500 V, 1 h at 1,000 V, and 5 h at 8,000 V for 18 cm and the same duration for 500 V and 1,000 V but 8 h at 8,000 V for 24 cm strips using Ettan IPGphor (GE Healthcare Biosciences, Pittsburgh, PA). The focused strips were first equilibrated immediately for 20 min in 7 ml per strip of SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) with 1% DTT and followed by a second equilibration for 20 min in 7 ml per strip of SDS equilibration buffer containing 2.5% IAA. After equilibration, IPG gel strips were embedded in a 1% agarose solution on top of the SDS-PAGE gel for second-dimension gel electrophoresis.

3.2.5 Second-dimension Gel Electrophoresis, Staining and Gel Analysis

SDS-polyacrylamide gels (12.5%), 235 x 190 x 1.5 mm (width x length x thickness) were prepared. SDS-PAGE was conducted at 22°C at a constant voltage of 110 V for 1608 Vh using

an Ettan Dalt 2-D electrophoresis system (GE Healthcare Biosciences, Pittsburgh, PA). Analytical gels were stained with Silver Stain Kit according to the manufacturer's instructions. Preparative gels were stained with 0.125% Coomassie brillant blue R-250 in 10% glacial acetic acid and 50% methanol followed by destaining in 10% acetic acid and 50% methanol. All stained gels were scanned using a UMAX PowerLook II scanner (UMAX data systems, Taiwan). The resulting 16-bit images were analyzed, and the changes of spot intensity between non-inoculated and inoculated samples were quantified using Progenesis Same Spots software (Nonlinear USA Inc, Durham, NC). Our preliminary comparisons of proteins from identical samples separated on different gels indicated that the average coefficient of variation of normalized volume of a given matched spot was 9.5%. Based on this, only those protein spots exhibiting at least a 1.5 fold change in intensity, when comparing 14 dai inoculated leaf samples against controls, were considered differentially expressed and chosen for peptide sequencing and further study in the time-course experiment.

3.2.6 Peptide Sequencing and Homology Analysis

Differentially expressed protein spots recovered and pooled from three Coomassie brilliant blue R 250-stained preparative two-dimensional (2-D) gels were subjected to in-gel trypsin digestion and *de novo* peptide sequencing using liquid chromatography tandem mass spectrometry (LC-MS/MS) at the Pennington Biomedical Center (Baton Rouge, LA). The *de novo* peptide sequences were compared to known protein and translated open reading frames of expressed sequence tags (ESTs) in the databases at the National Center for Biotechnology Information (NCBI) and SWISS-Prot using BLAST (Basic Local Alignment Search Tool) to identify their homologies (Altschul et al. 1997).

3.2.7 Data and Statistical Analysis

Statistical analysis of protein spot intensity in the time-course experiment was conducted using SAS (version 9.1; SAS Institute, Cary, NC). Significance of differences in fold change in protein spot intensity between inoculated and non-inoculated samples at the same time point was analyzed by the Student's *t*-test. Comparison of protein spot intensity between different time points for each spot was conducted by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test. In all case, $P \leq 0.05$ was used to determine statistically significant differences.

3.2.8 Cloning of cDNAs Encoding PR10 Protein.

Total RNA was extracted from the soybean leaf powder using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Extracted total RNA was treated with DNase I (Qiagen) to eliminate residual DNA contamination. Reverse transcriptase reactions were conducted with 6 µg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's protocol. Random hexamers from the kit reagents were used for the reaction. For PCR cloning of full coding sequence of *PR10*, 100 ng of the reverse transcribed cDNA was used. Degenerated primers were synthesized for spot 1, 5'-GA(A/G)AA(T/C)GT(I)GA(A/G)GG(I)AA(T/C)GG-3' (PR10-F) and 5'-(A)GT(T/C)TT(I)A(G/A)(T/C)TC(A/G)TC(T/C)TG(A/G)TT-3' (PR10-R), based on peptide sequences, ENVEGNG and NQDELKT, respectively. After the PCR reaction, PCR products were ligated to pCR2.1-TOPO vector (Invitrogen, Carlbad, CA) and sequenced. The remaining parts of the *PR10* gene were cloned by PCR using primers designed based on the DNA sequence of the PCR product. For cloning genomic DNA encoding PR10, the same primer sets for cDNA cloning were used. PCR amplification was conducted using genomic DNA extracted from soybean leaves using GenElute Plant Genomic DNA Miniprep Kit (Sigma, St. Louis, MO). PCR

product was directly ligated into the pCR2.1-TOPO vector, and three colonies were selected and sequenced. Resultant sequences were used for database homology search.

3.2.9 Total RNA Extraction and Real-time PCR Assay

Total RNA was extracted from the soybean leaves collected at various time points using RNeasy Plant Mini Kit and treated with DNase I to eliminate residual DNA contamination. Reverse transcription was conducted with 500 ng of total RNA using TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Real-time PCR assays were performed using 2x SYBR Green PCR Master Mix (Applied Biosystems) in 25 µl reaction volume with 1 µl of reverse transcribed cDNA, 12.5 µl of SYBR green, and 1 µM of each primer. ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used for real-time PCR under standard conditions. The same protocol was used for 18S rRNA (internal control) primers. Primers used for *PR10* and *CHI 1, 2, 3* were listed in Table 3.1. These specific primers were designed based on corresponding nucleotide sequences from databases (GU563345 for *PR10,* AF276302 for *CHI1*, DQ191404 for *CHI2*, and DQ 191405 for *CHI3*) (Table 3.1). Real time PCR for each *CHI* gene was conducted in this study to determine whether *CHI2* and *CHI3* gene were induced by ASR infection as *CHI1*. Melting curve analysis was conducted to confirm amplification of a single product and the absence of primer-dimers. Relative RNA expression of target genes was determined using ΔΔCt method ($[Ct_{(18S\ rRNA)} - Ct_{(target)}]_{inoculated} - [Ct_{(18S\ rRNA)} - Ct_{(target)}]_{control}$).

3.2.10 Chalcone Isomerase Activity Assay

Crude enzyme extract was prepared according to Mol et al. (1985). One gram of ground leaf powder was homogenized in a mortar with 1 g of Dowex (100-200 mesh) and 2 ml of 0.1 M sodium phosphate (pH 8.0) containing 1.4 mM ß-mercaptoethanol. The supernatant was recovered by centrifugation for 5 min at 12,000 rpm using an oak ridge centrifuge tube at 4°C.

Supernatant was homogenized twice, each with 0.5 g Dowex (100-200 mesh), to remove residual flavonoids. The final recovered yellowish supernatant was used as a crude enzyme extract. Enzyme assay was conducted according to van Weely et al. (1983). The reaction was started by addition of isoliquiritigenin (2', 4', 4', 6'- tetrahydroxychalcone) to a final concentration of 18.4 µM in a 1 ml reaction mixture (0.1 M potassium phosphate, pH 7.5, 10 mM KCN, and 10 mM ethylene glycol monomethyl ether (2-methoxy ethanol). Non-enzymatic cyclization was monitored for 1-2 min by measuring the decrease in absorbance at 365 nm before the addition of a crude enzyme extract. The enzymatic cyclization was then monitored after addition of 25 µl crude enzyme extract. The non-enzymatic conversion measured in the first 2 min was subtracted from the total conversion measured after the addition of enzyme for correction. The assay was conducted three times and the mean activity was used for comparison.

Table 3.1. Primer sets designed for real–time polymerase chain reaction.

Genebank ID	Target gene	Orientation	Primer sequence (5'-3')	Amplicon size (bp)
AF276302	Chalcone isomerase1	Forward	AATTTTGCACCTGGTGCCTC	106
		Reverse	TCACTGCAGCCTCCTTTTCTG	
DQ191404	Chalcone isomerase2	Forward	AAATTTCCCACCAGGCTCCA	131
		Reverse	CCTCCGAAAGTGGCTTGTTGT	
DQ191405	Chalcone isomerase3	Forward	CCAGTTAACGGAATCCGACCA	133
		Reverse	GGCCACACAATTTTCTGCCA	
GU563345	*PR10	Forward	AAATCAACTCCCCTGTGGCTC	121
		Reverse	CCACCATTTCCCTCAACGTTT	

*; soybean PR10 genomic DNA was cloned in this study, and sequence was submitted to the Genebank (GU563345).

3.3 Results

3.3.1 Comparison of Protein Profiles between Non-inoculated and Inoculated Soybean Leaf in Different pH Ranges

The non-inoculated and inoculated soybean leaf samples contained 1436 ± 138 and 2034 ± 67 protein spots, respectively, when resolved using 18 cm, pH 3 to 10 NL IPG strips for the first dimension and 12.5% SDS-PAGE gels for the second dimension (Fig 3.1, A and B).

To further enhance protein separation in the region concentrated with protein spots between pH 4 to 7, a narrower pH 4 to 7 IPG strip was used (Fig 3.1, C and D). A total of 1202 ± 4 and 1598 ± 17 protein spots were detected in the pH 4 to 7 2-D gels containing the non-inoculated and inoculated soybean leaf samples, respectively. This represents an increase of 133 and 379 more spots when compared to 1069 ± 45 and 1129 ± 50 spots detected from the same region of pH 3 to 10 NL gels for the non-inoculated and inoculated leaf samples, respectively. Spot image intensity analysis of leaf protein samples separated using pH 3 to10 NL gels identified 11 up-regulated and three down-regulated (≥ 1.5 fold) (Fig. 3.1; spots 13, 38 and 39) spots in the inoculated samples. Analysis of protein samples separated using pH 4 to 7 gels identified 26 additional differentially expressed protein spots (≥ 1.5 fold) between inoculated and non-inoculated leaves: 21 spots up-regulated and 5 down-regulated (spots 6, 11, 12, 13, and 28) in the inoculated soybean leaves (Fig. 3.1). Spot 1 showed the highest induction of 21-fold whereas spot 13 showed the highest suppression of five-fold in inoculated leaves compared to their corresponding protein spot in the non-inoculated leaves. Changes of these protein spots were observed reproducibly in three biological repeats.

3.3.2 Identification of Differentially Expressed Protein Spots

Eleven up-regulated and 3 down-regulated protein spots that were visible in Coomassie brillant blue stained gels were recovered and sequenced (Table 3.2). Ten of the differentially expressed spots were found in both gels (pH 3 to 10 NL and pH 4 to 7) and the other four spots were only found in pH 7 to 10 region of pH 3 to 10 NL gels. Peptide sequences of each spot and their sequence homology identified through database searches were summarized in Table 3.2. Peptide sequences of spot 1 showed 100% match to a PR 10-like protein (AF529303) (Chou et al. 2004) and a SAM22 protein (Crowell et al. 1992) from *Glycine max*. It also showed high sequence similarity to PR 10 protein from other plants, such as *Vigna unguiculata*, and

Lupinus albus (Fig. 3.2). Spot 2 showed high sequence similarity to the deduced amino acid sequences of a stress induced gene H4 (X60044) from *G. max*. It is interesting to point out that peptide sequences between spots 1 and 2 shared 48% identity.

The peptide sequences from Spot 8 showed a complete match to *G. max* chalcone isomerase 1 (CHI1, AF276302) (Cramer et al. 1985; Lambais and Mehdy 1993; Seehaus and Tenhaken 1998; van de Mortel et al. 2007). It also showed 32% and 31% identity to CHI2 and CHI3, respectively (Fig. 3.3). Spots 16, 36, and 40 showed high homology to a chloroplast L-ascorbate peroxidase from *Oryzae sativa*, a stress inducible protein from *G. max*, and a β-1,3 glucanase from *G. max* (Keen and Yoshikawa 1983; Lambais and Mehdy 1993), respectively.

Spot 26 was identified as a *G. max* cytosolic glutamine synthetase, and spot 32 showed high homology to the S-adenosylmethionine synthetase I from *Oryza sativa* based on their sequence (Table 3.2). Spots 13, 38 and 39 all showed high homology to a 28/31 kDa glycoprotein (or vegetative storage protein) from *G. max*. The five peptide sequences obtained from spot 38 were identical to five of the nine peptides obtained for spot 39. The other four peptides also matched to the same glycoprotein (Table 3.2). Spot 14 was highly homologous to an α-soluble ethylmaleimide sensitive attachment protein from *Helianthus annuus* (sunflower) that functions in protein modification and transportation (Subramaniam et al. 1997). Spot 19 showed 83% homology to a 3'5'-cyclic phosphodiesterase from *Trichomonas vaginalis*, indicating it is likely a protein of rust origin. The peptide sequences from spot 37 showed 100% match to cyclophilin/peptidyl-prolyl cis-trans isomerase from maize (ACG31960) and cotton (ACT63839). It also showed significant homology (92%) to a yeast (*Schizosaccharomyces pombe*: NP_595664.1) cyclophilin, which is involved in protein folding (Wang et al. 2001).

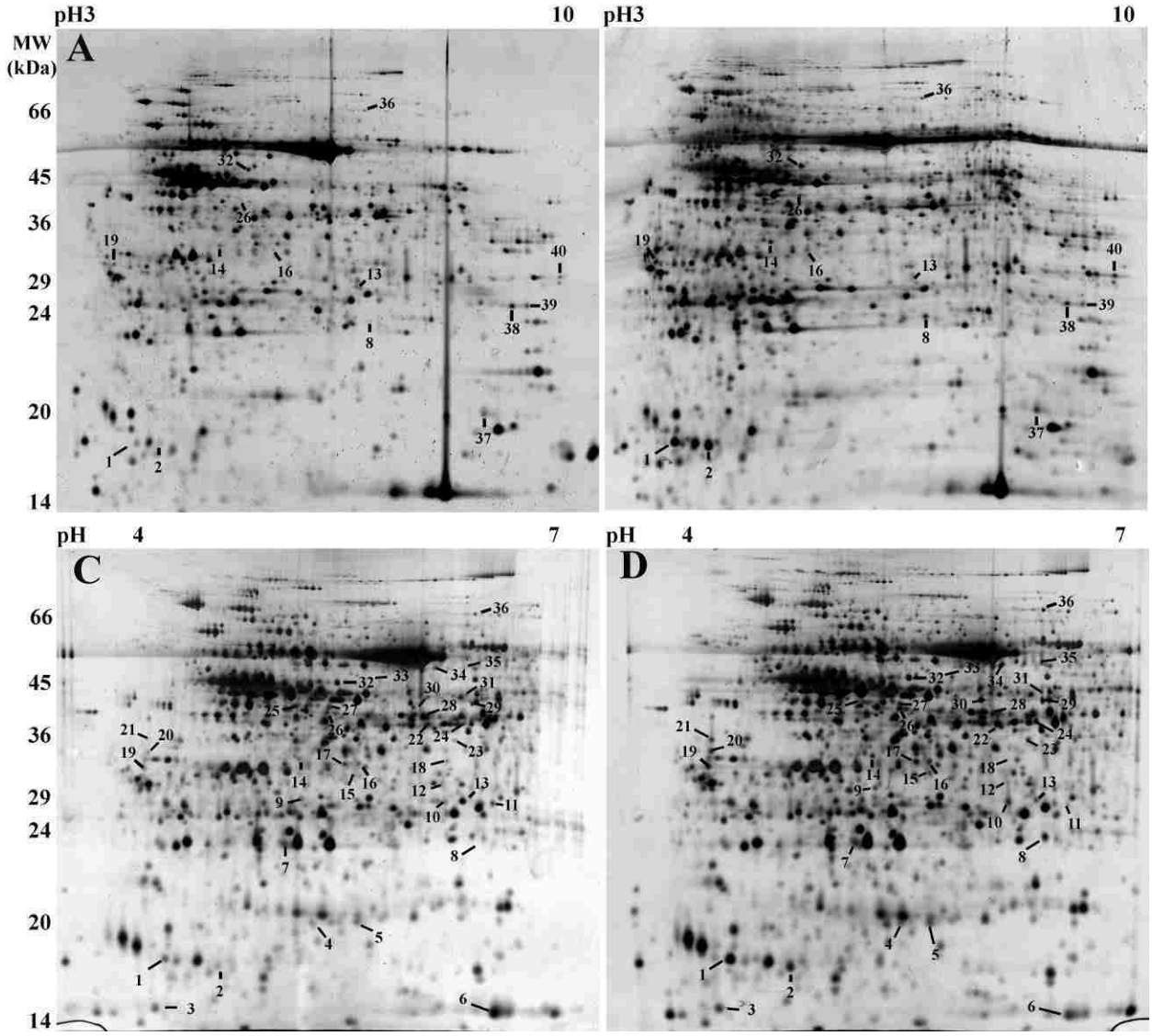


Figure 3.1. Comparison of non-inoculated and inoculated soybean leaf two-dimensional (2-D) protein gel images 2 weeks after infection. The down-regulated protein spots were indicated with asterisk and the rest of the spots without asterisk were up-regulated. Non-inoculated (water-inoculated, A and C) and rust-inoculated (B and D) leaf proteins were separated on pH 3 to 10 NL (A and B) or pH 4 to 7 (C and D) gradient strips.

Table 3.2. Identification of differentially expressed protein spots from soybean leaves inoculated with *Phakopsora pachyrhizi* through peptide sequencing using tandem mass spectrometry (MS/MS).

Spot	Top Hit	Organism	GenBank	pI	MW (kDa)	e-value	Coverage (%)	Peptide sequence
1	PR10-like protein	*G.max*	AF529303	4.46	17.41	5e-22	54	KALVTDADNVIPKA KSVENVEGNGGPGTIKK KITFLEDGETKF KGDAEPNQDELKT KAIEAYLLAHPDYN
2	Stress-induced gene (H4)	*G.max*	X60044	4.65	18.23	2e-9	41	TFEDETTSPVAPATLYK LAVTDAGSLALPK SVENLEGNGGPTGLK QDQPNPDDLK AVEAYLLANPHYN LTFVEDGESK
8	Chalcone--flavonone isomerase	*G.max*	AF276302	7.17	23.10	2-23	33	RTYFLGGAGERG KFTGIGVYLEDKA RDIISGPFEKL KSVGTYGDAEAAAIEKF KAVSAAVLETMIGEHAV SPDLKR RLPAVLSHGIIV
13*	31 kDa glycoprotein	*G.max*	P10743	7.07	25.95	3e-18	31	KTIPEECVEPT KDYINGEQFRS KGDAPALPETLKN KMAVTEANLKK AGFHTWEQLILKD RIVGIIGDQWSDLLGDHRG
14	alpha-soluble N-nsf attachment protein	*R. communis*	XP_002522820	4.93	34.24	4e-11	20	YEDAADLFDK KVAQFAAQLEQYQK AMEIFEEIA RYQDLDPTFSGTR EFDSMTPLDSWK LNGYGIFGSK
16	Probable chloroplast L-ascorbate peroxidase	*N. nucifera*	ABO21422	6.83	33.48	5e-16	21	RLGWHDAGTYNKN KHAANAGLVNALKL KEIVALSGAHTLGRS TGPGAPGGQSWTVQWL KFDNSYFKD KYAEDQEAFFKD
19	3'5'-cyclic nucleotide phosphodiesterase family protein	*T. vaginalis*	xp_001310604	5.6	124	47	1	NLNSVKQSNLQVK
26	Cytosolic glutamine synthetase	*G.max*	AF301590	5.96	40.81	2e-21	29	KVIAEYIWIGGSGMDLRS RTLPGPVSDPSELPKW RGNNILVICDAYTPAGEPI PTNKRH RDIVDAHYKA KGDWNGAGAHTNYSTKT REDGGYEVIKA RHETADINTFLWGVANRG KEHIAAYGEGNERR

Table 3.2. Continued

Spot	Top Hit	Organism	GenBank	pI	MW (kDa)	e-value	Coverage (%)	Peptide sequences
32	S-adenosylmethionine synthetase 1	*C. arietinum*	EU924160	5.41	43-.13	8e-70	45	METFLFTSESVNEGHPDK LCDQISDAVLDACLEQDP DSK TNMVMVFGEITTK NIGFVSDDVGLDADNCK VLVNIEQQSPDIAQGVHG HL TK TQVTVEYYNDK VHTVLISTQHDETVTNDEI AADL KEHVIKPVIPEKTIFHLNPS GR FVIGGPHGDAGLTGR TAAYGHFGR DDADFTWEVVKPLK
36	*S*tress inducible protein (*Sti*)	*G. max*	Q43468	5.74	63.54	2e-07	5	KALELDDEDISYLTNRA KELEQQEYFDPKL
37	Cyclophilin, Peptidyl prolyl cis trans isomerase	*Z. mays*	ACG31960	8.95	18.34	2e-07	10	KHVVFGQVVEGMDVVKA
38*	Stem 31 kDa glycoprotein	*G. max*	P10742	8.91	24.66	4e-10	18	KEYIHGEQYRS KTVNQQAYFYARD KGNAPALPETLKN KDPQDPSTPNAVSYKT KIIFLSGRT
39*	Stem 31 kDa glycoprotein or vegetative storage protein	*G. max*	P 10742	8.86	29.37	8e-24	29	KEYIHGEQYRS KTVNQQAYFYARD KFNSTLYDEWVNKG KGNAPALPETLKN KIIFLSGRT KDPQDPSTPNAVSYKT RGESRTFKL
40	β-1,3-endoglucanase	*G. max*	Q03773	9.13	31.63	2e-05	10	KVSTAIDTGALAESFPPSK GRSPSVVVQDGSLGYRN

*: indicate spots that were down-regulated in inoculated soybean leaves; the rest of spots were up-regulated in inoculated soybean leaves

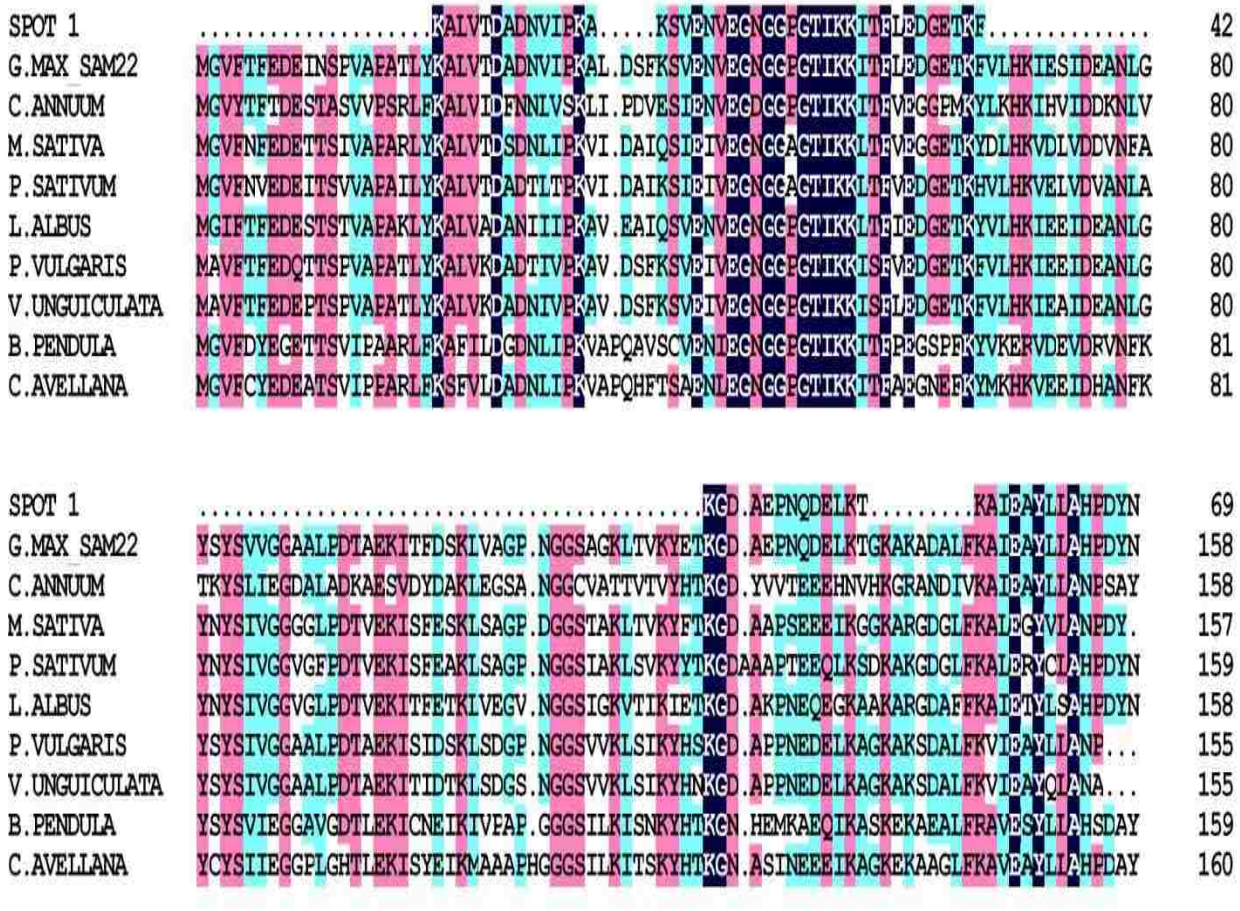


Figure 3.2. Homology comparison between peptide sequences from spot 1 and deduced amino acid sequences of *PR10* from soybean and other plant species. SPOT1, sequenced in this study; *Glycine max*_SAM22, X60043; *Capsicum annuum*, AY829648; *Medicago sativa*, X98867; *Pisum sativum*, M18249; *Lupinus albus*, AJ000108; *Phaseolus vulgaris*, X61364; *Vigna unguiculata*, AB027154; *Betula pendula*, AJ289771; and *Corylus avellana*, AF136945.

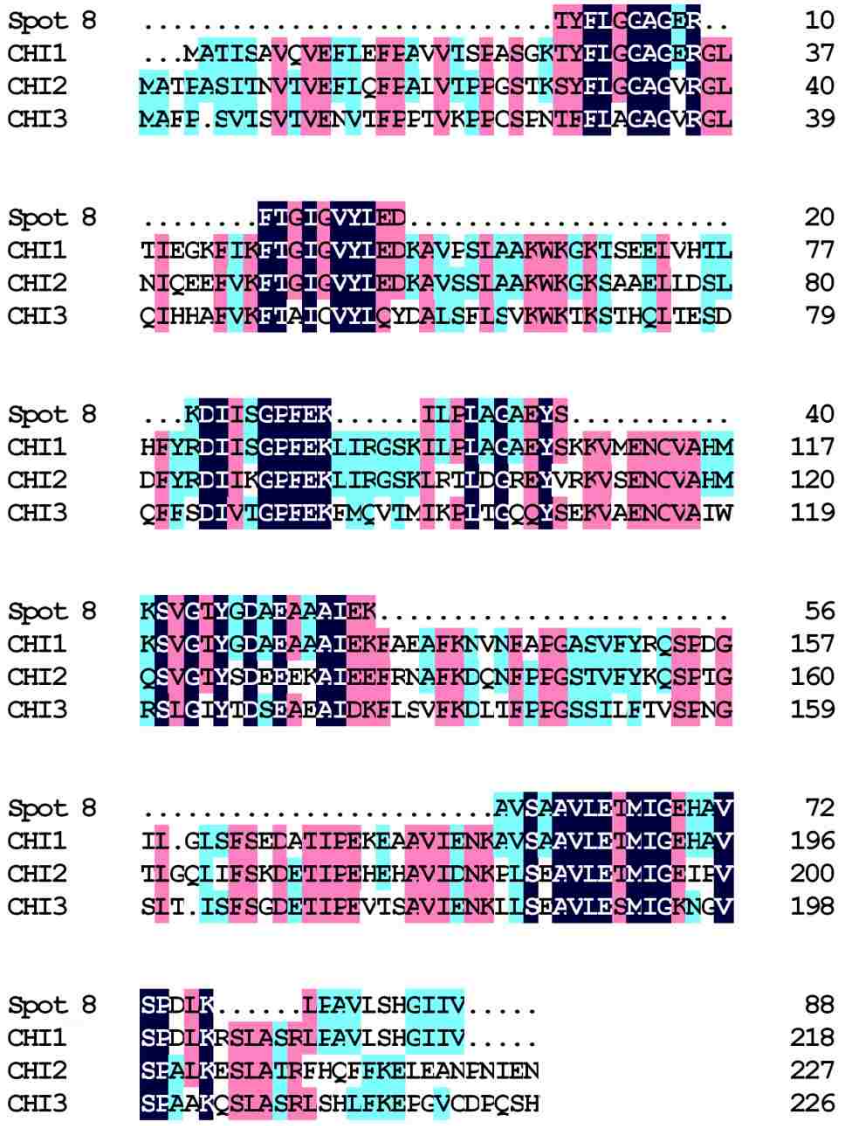


Figure 3.3. Amino acid sequence comparison of spot 8. Alignment of the amino acid sequence of spot 8 with deduced peptide sequences of *CHI1*, *CHI2*, and *CHI3* genes from *Glycine max*.

3.3.3 Changes in Production of Identified Protein Spots after *P. pachyrhizi* Infection during the Time-course

The expression of the 14 sequenced protein spots that showed over 1.5-fold increase or decrease in protein levels in the inoculated soybean leaves 14 dai were further examined to determine how these proteins respond during the time-course of rust infection. Relative fold change for each of these differentially expressed proteins normalized to the same protein spot

from the non-inoculated control over the time-course is shown in Table 3.3. Protein spots, 1, 2, 8, 16, 36, and 40 involved in plant defense or stress were up-regulated. The highest induction for most of these protein spots was detected at either 6 or 8 dai. Spots 1 (PR10 protein), 16 (chloroplast L-ascorbate peroxidase) and 36 (stress inducible protein) responded quickly to soybean rust infection at 10 hai with 10, 2 and 1.6-fold increase, respectively, compared to non-inoculated control. The production of PR10 (spot 1) was significantly induced up to 6 dai compared to the control, which showed a low basal expression during the time-course (Fig 3.4). The expression pattern of spot 2 (a stress-inducible gene, H4) was different from that of spot 1. Spot 2 was actually down-regulated at 10 hai and became significantly induced at 12 dai (2-fold). However, the earliest significant induction after rust infection for spot 8 (CHI1) was detected at 6 dai compared to control, which also showed a very low basal level expression of CHI1 (Fig. 3.4). Spots 36 (stress inducible protein) and 40 (β-1,3-glucanase) were significantly induced (2-fold) at 14 and 12 dai, respectively. This slow induction was possibly an indirect or secondary result of rust infection.

Protein spots belonging to plant metabolism or growth and development responded differently to soybean rust infection compared to the defense related proteins. Spots 13, 16, and 38 (all glycoproteins) were reduced by over 2-fold at 12 dai. Spot 14, involved in protein modification and transformation, was induced by 1.7 fold at 10 hai and increased gradually to about 3.3 fold at 14 dai. Spot 26 (glutamine synthetase) showed a significant induction (ranging from 2.7 to 6.8 fold) during the entire time-course. In contrast, spot 32 (S-adenosylmethionine synthetase I) was down-regulated initially, but was significantly induced at 4 and 14 dai. Spot 37 showed a pattern of induction similar to spot 40, which was only significantly induced at a late stage of infection. Spot 19, a potential fungal protein, was significantly induced from 2 dai. Overall, five proteins (PR10, alpha-soluble N-ethylmaleimide-sensitive attachment protein,

ascorbate peroxidase, glutamine synthetase, and a stress inducible protein) were induced rapidly by soybean rust infection at as early as 10 hai and the rest of the proteins responded more slowly over the time-course of the experiment.

3.3.4 Cloning of cDNAs and Genomic DNA Encoding PR10

The time-course experiment showed that spot 1 (PR10 protein) was induced earlier and to higher levels after soybean rust infection than any other protein spot, and spot 8 (chalcone isomerase I), a known plant defense related protein, was also induced during early stages of rust infection. To further study their possible roles in soybean response to rust infection, cDNA and genomic DNA of these two proteins were cloned. PCR amplification with degenerate primers corresponding to spot 1 generated a 281 bp product (Fig. 3.5). The DNA sequence of this PCR product showed 100% homology to a gene encoding a *G. max* stress inducible protein (SAM22) (X60043), but low homology (63%) to the PR10-like protein (AF529303, contains ins/del compared to X60043) (Supplement 2) because of two separated deletions. The cloned full-length coding sequence (477 bp) of PR10 showed 99% homology with the stress inducible protein (SAM22) (X60043) (Fig. 3.6). The cloned genomic DNA (634bp, GenBank accession: GU563345), which contained an intron of 157 bp, also showed near 100% homology to the stress inducible protein (SAM22) (X60043) in the exon regions with only one nucleotide disagreement in the second exon region (Fig. 3.6). Homology search using the nucleotide sequences of X60043 resulted in 100% similarity with a partial sequence of soybean PR10 (DQ267260; Graham et al. 2003) (data not shown). Based on these sequence data, spot 1 is concluded to be a PR10 protein. Since spots 1 and 2 showed high amino acid sequence similarity and the positions of these spots on the 2-D gel were very close (similar pI and MW), they could be different isoforms of PR10, which has been previously reported (Bestel-Corre et al. 2002; Xie et al. 2010).

Table 3.3. Relative fold change vs. control samples at each time point from soybean leaf during *Phakopsora pachyrhizi* infection. (Proteins induced by rust infection were normalized by the proteins induced during the developmental process in non-inoculated leaves at each time point).

Spot	**Identity**	Fold change at time[a]									
		10h	1d	2d	3d	4d	6d	8d	10d	12d	14d
1	PR10-like protein	10.4±1.2 (0.004)	10.6±2.6 (0.0002)	12.2±2.9 (<.0001)	13.3±1.6 (0.0004)	8.8±2.2 (0.041)	15.2±2.0 (0.012)	24±2.2¶ (0.006)	15±3.3 (0.013)	18.3±2.4 (<.0001)	21.2±3.4 (<.0001)
2	Stress-induced gene (H4) or PR10 –like protein	0.6±0.18 (0.025)	0.7±0.04 (0.022)	0.9±0.02 (0.752)	1.1±0.5 (0.687)	1.3±0.3 (0.524)	1.5±0.5 (0.128)	1.8±0.5 (0.063)	1.8±0.1 (0.072)	2.0±0.02¶ (0.020)	2.0±0.2 (0.002)
8	Chalcone--flavonone isomerase	1.4±0.3 (0.092)	1.2±0.1 (0.215)	1.2±0.2 (0.654)	1.4±0.4 (0.321)	2.1±0.1 (0.236)_	1.8±0.3 (0.012)	2.9±0.3¶ (0.021)	2.2±0.1 (0.015)	2.8±0.1¶ (<.0.0001)	2.6±0. (<.0.0001)
13*	Soybean 31 kDa glycoprotein	0.8±0.4 (0.072)	0.9±0.5 (0.089)	1±0.3 (0.091)	1±0.6 (0.096)	0.8±0.3 (0.082)	0.9±0.3 (0.051)	0.9±0.2 (0.066)	0.7±0.3 (0.005)	0.2±0.04¶ (0.019)	0.2±0.08¶ (0.043)
14	alpha-soluble N-ethylmaleimide-sensitive attachment protein	1.7±0.4 (0.050)	1.4±0.5 (0.084)	1.4±0.6 (0.052)	1.8±0.5 (0.017)	2.3±0.7 (0.028)	2.2±0.5 (0.035)	2.8±0.5 (0.045)	2.5±0.6 (0.029)	2.8±0.04 (0.041)	3.3±0.3¶ (0.012)
16	Probable chloroplast L-ascorbate peroxidase (OsAPx06)	2.1±0.4 (0.021)	3.6±0.8 (0.023)	1.5±0.4 (0.041)	2.9±0.05 (0.014)	0.7±0.2 (0.035)	5.2±0.8¶ (0.016)	2.2±0.1 (0.018)	1±0.5 (0.723)	2.1±1.7 (0.032)	2.0±0.4 (0.021)
19	3'5'-cyclic nucleotide phosphodiesterase family protein	1±0.4 (0.432)	1.2±0.5 (0.743)	1.6±0.5 (0.450)	2.3±0.2 (0.256)	1.9±0.3 (0.478)	1.6±0.8 (0.152)	3.9±0.3 (0.271)	5.5±2.2¶ (0.007)	2.7±0.1 (0.041)	3.9±1 (0.017)
26	cytosolic glutamine synthetase	5.8±1.4 (0.0002)	4.1±0.7 (0.012)	5.0±0.4 (0.003)	3.6±1.3 (0.006)	2.7±0.4 (0.012)	4.3±1 (0.001)	4.7±0.3 (0.006)	4.5±0.2 (0.004)	6.8±0.5¶ (0.005)	6.5±1 (<.0.0001)
32	S-adenosylmethionine synthetase 1	0.7±0.4 (0.001)	0.8±0.2 (0.009)	0.7±0.3 (0.001)	0.7±0.2 (0.003)	1.8±0.3¶ (0.002)	0.9±0.7 (0.001)	1.5±0.1 (0.005)	1.4±0.1 (0.047)	1.3±0.4 (0.051)	2.0±0.3¶ (0.006)
36	*Sti* gene; stress inducible protein	1.6±0.1 (0.012)	1.2±0.1 (0.001)	1.9±0.2 (0.321)	1.5±0.09 (0.012)	1.5±0.05 (0.021)	1.7±0.3 (0.158)	1.7±0.08 (0.385)	1.8±0.02 (0.147)	1.7±0.01 (0.721)	1.9±0.1¶ (0.052)
37	Cyclophilin, Peptidyl prolyl cis trans isomerase	1.0 ±0.2 (0.953)	1.1±0.2 (0.715)	1.3±0.8 (0.624)	1.3±0.4 (0.180)	1.3±0.3 (0.157)	1.0±0.1 (0.625)	1.1±0.1 (0.432)	1.2±0.2 (0.351)	1.5±0.3 (0.147)	2±0.2¶ (0.008)
38*	Stem 31 kDa glycoprotein precursor	0.8±0.4 (0.326)	0.9±0.12 (0.621)	1.15±0.5 (0.426)	0.7±0.2 (0.157)	0.3±0.2 (0.189)	0.9±0.2 (0.821)	0.3±0.01 (0.100)	0.7±0.3 (0.042)	0.4±0.0¶ (0.026)	0.5±0.1 (0.048)
39*	Soybean 28/31 kD glycoprotein or vegetative storage protein	1.4±0.5 (0.284)	0.9±0.1 (0.815)	0.9±0.5 (0.473)	0.8±0.3 (0.382)	0.4±0.03 (0.317)	1.0±0.3 (0.972)	0.4±0.03 (0.132)	0.8±0.2 (0.421)	0.3±0.02¶ (0.045)	0.7±0.1 (0.024)
40	Soybean β-1,3-endoglucanase	0.9±0.4 (0.046)	1.3±0.2 (0.089)	1.1±0.4 (0.050)	1.0±0.8 (0.047)	1.2±0.3 (0.039)	1.1±0.2 (0.052)	0.8±0.2 (0.049)	1.5±0.1 (0.052)	2.0±0.1 (0.038)	2.3±0.3¶ (0.017)

[a] Relative fold-change vs. control samples at each time point after inoculation [mean ±standard deviation(*P* value)]. The Student's *t* test was used to find whether there was significant difference ($p<0.05$) between non-inoculated and inoculated soybean leaves. The relative fold-changes are means from three different experiments.

*: indicates spots that were down-regulated in inoculated soybean leaves; the rest of spots were up-regulated in inoculated soybean leaves.

¶ represents significant fold-change during the time course. LSD test was used for statistical analysis at $P=0.05$

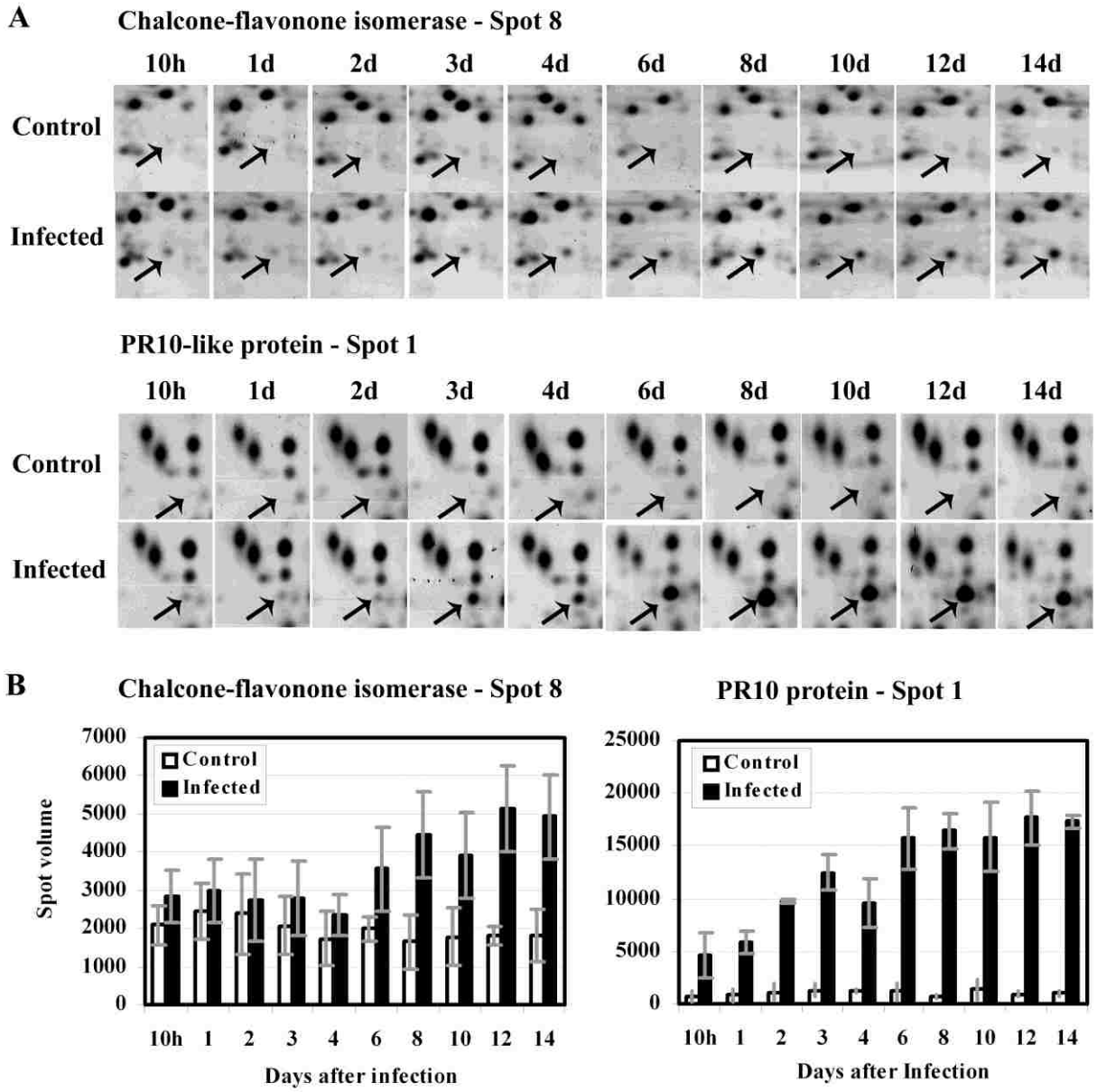


Figure 3.4. Changes of spot 8 (CHI1) and spot 1 (PR10) protein level during the time-course. A, sub-sections of the 2-D gel of protein spots 8 and 1; B, volume change of spots 8 and 1 from control and infected during the time-course. The bar graphs show average volume for the pointed spots with standard deviations.

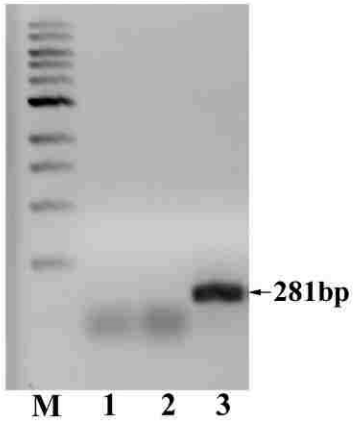


Figure 3.5. PCR amplification of *PR10* product using degenerated primers synthesized based on spot 1 peptide sequences identified by tandom MS spectrometery. M, marker; 1, no template; 2, no primers; and 3, cDNA template.

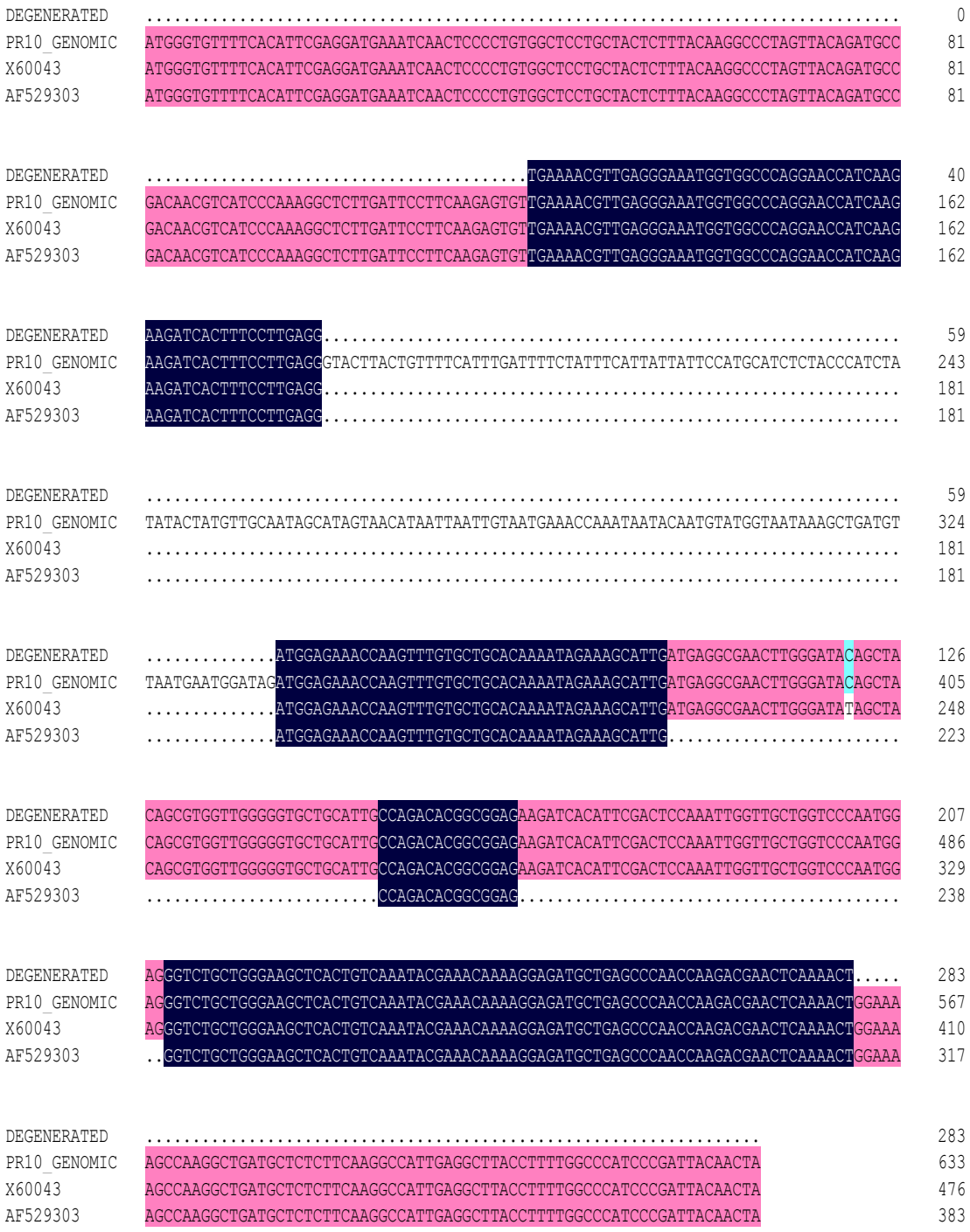


Figure 3.6. *PR10* DNA sequence comparisons between cDNA cloned by PCR, PR10 genomic DNA cloned in this study (GU563345), AF529303 (PR10 like protein), and X60043 (stress inducible gene, SAM22).

3.3.5 Changes of Chalcone Isomerase 1 and PR10 at Transcript Level during the Time-course of Rust Infection

The corresponding transcripts of chalcone flavonone isomerase and PR10 were examined using real time PCR to determine whether their changes follow the same pattern as those at the protein level and to find the time point of gene expression initiation by ASR infection. In non-inoculated control leaves, the basal expression of *CHI1* gene was much higher than that of either *CHI2* or *CHI3* (Fig. 3.7 A) and the abundance of *CHI3* was the lowest among the three at all time points. The relative abundance of *CHI1* was dramatically increased by the ASR infection and was significantly higher than *CHI2* and *CHI3* in most of the time points except 1, 4, or 6 dai when *CHI2* and/or *CHI3* showed a level of expression close to *CHI1* (Fig. 3.7 B). When comparing the transcript levels between infected and control samples, all three *CHI* genes showed significant induction by ASR infection compared to control leaves at 10 hai, but the magnitude of induction for *CHI1* (about 30 fold) was much higher than that for *CHI2* or *CHI3* (about 10-15 fold) (Fig. 3.8). *CHI1* transcript levels were reduced drastically to the level of the non-inoculated control at 1 dai. It was induced thereafter and peaked at 8 dai, but the induction was about 1/3 of that seen at 10 hai. *CHI2* and *CHI3* expression followed a similar pattern of induction and peaked at 6 dai and the induction for *CHI3* expression remained high (10-23 fold) up to 12 dai (Fig. 3.8). Although the relative expression ratio of *CHI3* was significantly higher than that of *CHI1* from 6 to 12 dai, the abundance of *CHI3* was significantly lower in non-infected and infected compared to that of *CHI1* (Fig. 3.7 A and B). The highest induction of *PR10* expression (38 fold) in inoculated leaves was detected at 10 hai compared to that in non-inoculated control leaves. It decreased to that of non-inoculated control leaves at 1 dai. Its expression was induced thereafter and peaked at 6 dai, but its expression was not as high as at 10 hai (Fig. 3.9). In addition, a low basal level expression of *PR10* was detected at all time points in non-inoculated control leaves (Fig. 3.10).

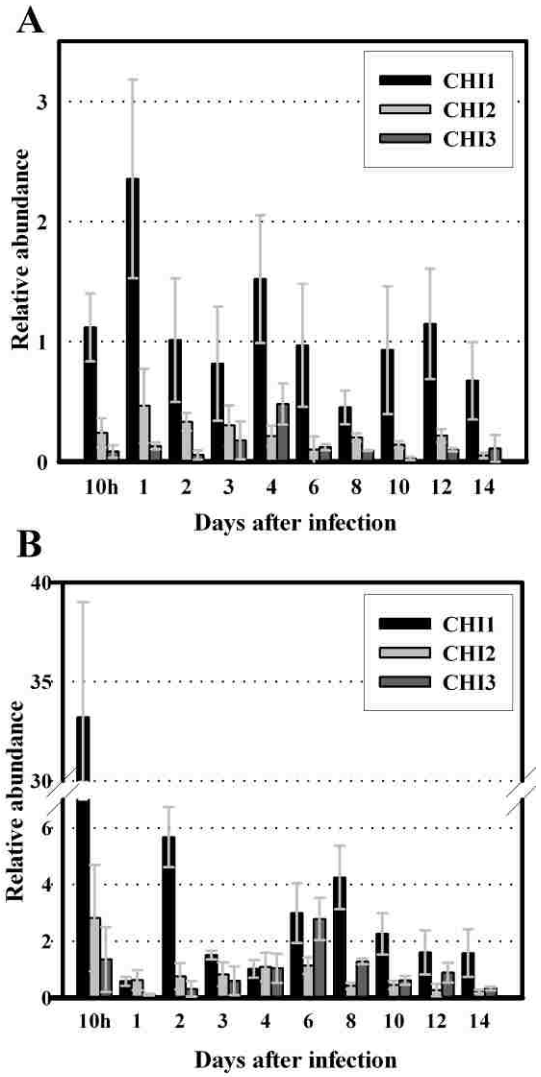


Figure 3.7. Comparison of *CHI1*, *CHI2*, and *CHI3* gene expression using quantitative real-time PCR after and befor soybean rust infection during the time-course. A, Control (non-infected); B, Infected. Relative abundance represents gene expression level normalized by the level of the 18s rRNA gene expression at each time point. The bar graphs show mean of relative gene expressions with standard deviations.

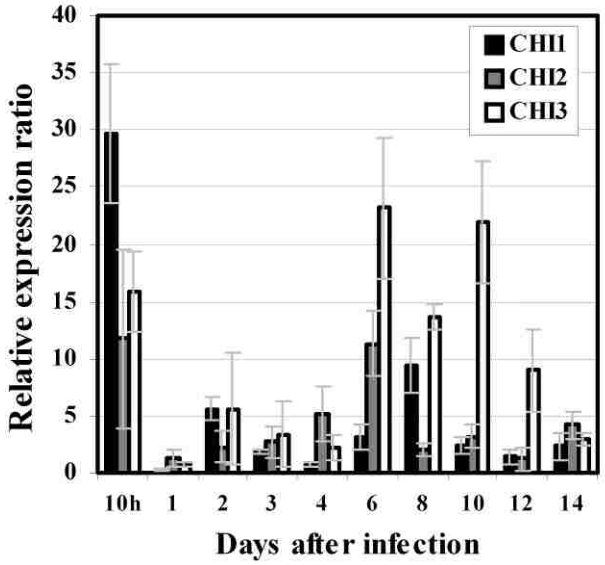


Figure 3.8. Comparison of *CHI1*, *CHI2*, and *CHI3* gene expressions using quantitative real-time PCR after soybean rust infection during the time-course. The ratio of gene expression changed after infection when each time point was calculated by normalization to the gene expression of the non-infected at the same time point. The bar graphs show mean relative expression ratios with standard deviations.

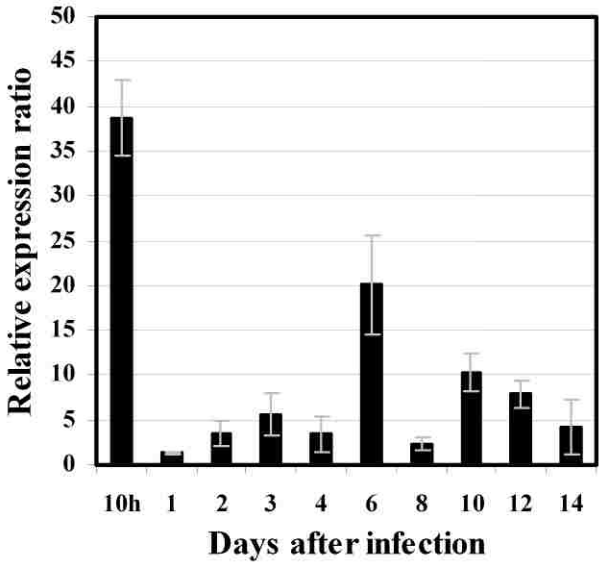


Figure 3.9. Comparison of gene expression of PR10 using quantitative real-time PCR after soybean rust infection during the time-course. The ratio of the gene expression changed after infection when each time point was calculated by normalization to the gene expression of the non-infected at the same time point. The bar graphs show mean relative expression ratios with standard deviations.

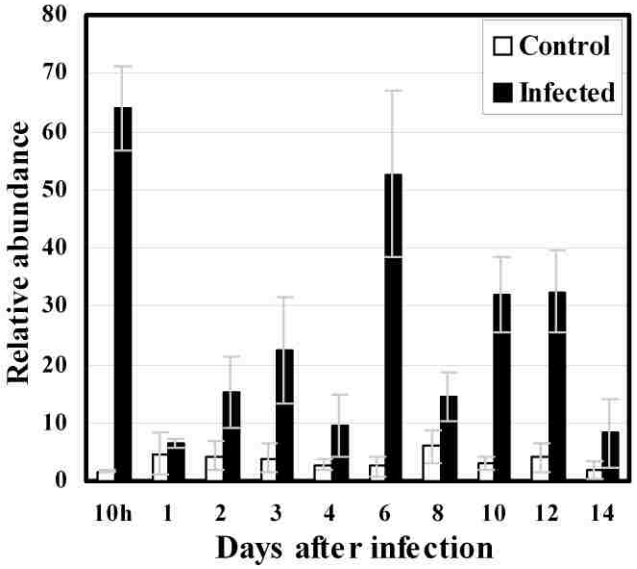


Figure 3.10. Comparison of *PR10* gene expression between control (non-infected) and infected soybean leave using quantitative real-time PCR during the time-course. Relative abundance represents gene expression level normalized by the level of the 18s rRNA gene expression at each time point. The bar graphs show mean relative expression abundance with standard deviations.

3.3.6 Changes of Chalcone Isomerase Enzyme Activities during the Time-course after Rust Infection

The enzyme activity of CHI was compared between non-inoculated and inoculated leaves during the time-course to study whether CHI protein volume changes detected by proteomics have a similar pattern with CHI enzyme activity. CHI in non-inoculated showed a constitutive basal enzyme activity throughout the time points (Fig. 3.11). The CHI activity increased gradually and peaked at 6 dai in infected leaves. The induction in enzyme activity in inoculated leaves was significant at all time points compared to non-inoculated control (p=0.05) (Fig. 3.11). CHI enzyme activity from 10 hai to 4 dai didn't follow the protein changes of CHI1 but they showed similar changes to each other from 6 dai.

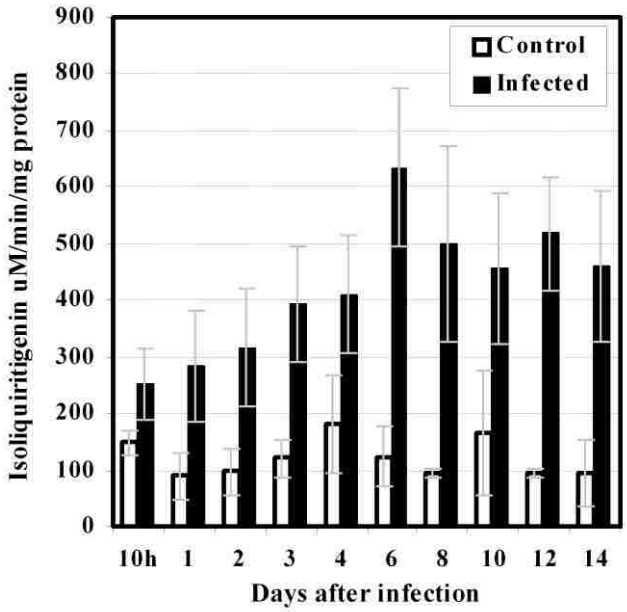


Figure 3.11. Comparison of enzyme activity of CHI between non-inoculated and inoculated soybean leaves during the time-course. The bars represent the mean enzyme activity with standard deviations.

3.4 Discussion

3.4.1 Improved Protein Isolation and Separation were Achieved through a Phenol-Based Protein Extraction and Using a Narrower pH Gradient

Several methods have been used to extract proteins from soybean leaves for proteomics (Ahsan and Komatsu 2009; Sarma et al. 2008; Xu et al. 2006). In a preliminary study, we compared two of them: TCA/acetone method (Granier 1988) and phenol-methanol method (Hurkman and Tanaka 1986). The phenol-methanol method consistently produced highly resolved 2-DE gels. This method also produced highly resolved 2-D protein profiles of other plants or plant tissues (Carpentier et al. 2005; Saravanan and Rose 2004). It is believed that phenol extraction and methanol precipitation can effectively reduce contamination of protein extracts with rigid plant cell wall, membrane lipids, and nucleic acids, which impede protein solubility and electrophoresis conductivity (Saravanan and Rose 2004). Our initial studies using pH 3 to 10 NL gradient for the first dimension found that the majority of the protein spots were

located between pH 4 to 7, therefore, pH 4 to 7 gradient was also used in this study, which resulted in a significant (13 to 33%) increase in the number of new spots/proteins detected due to better resolution.

3.4.2 Identification of Differentially Expressed Proteins after *P. pachyrhizi* Inoculation and Their Possible Functions through Sequence Homology Analysis

In this study, 40 protein spots were found differentially expressed after rust inoculation, and 14 spots were recovered and sequenced. Based on peptide sequence homology analysis, these differentially expressed proteins belong to four different groups: plant defense or stress related proteins; plant metabolism, growth and development; protein modification or transport; and rust fungal proteins.

Proteins belonging to the first group include PR10 protein (spots 1), CHI1 (spot 8), chloroplast ascorbate peroxidase (spot 16), stress inducible proteins (spots 2 and 36), and β-1,3-endoglucanase (spot 40). It is interesting to point out that the proteins in this group were all up-regulated in soybean leaves after rust inoculation. Induction of *PR10* during pathogen attack of plants has been previously reported in *Medicago truncatula* and *Zea mays* (Bestel-Corre et al. 2002, Xie et al. 2010). Reduced expression of *PR10* through RNAi gene silencing also leads to increased susceptibility of maize to *Aspergillus flavus* infection (Chen et al. 2010). Decreased expression of L-ascorbate peroxidase (spot 16) resulted in elevated level of H_2O_2 and a hypersensitive reaction (HR) in resistant tomato following avr5 elicitor treatment (Vera-Estrella et al. 1994). Ascorbate peroxidase also was inhibited by salicylic acid (SA) treatment, which induces a signaling pathway to activate resistance genes in tobacco (Durner and Klessing 1995). This agrees with our proteomics study, which showed that ascorbate peroxidase levels were induced after rust infection in this susceptible (compatible) interaction. A soybean stress inducible protein (spot 36) was moderately induced in this susceptible interaction. Its homolog

from *G. max* was highly induced by heat and cold treatment (Torres et al. 1995). This protein contains a TRP (tetratricopeptide-repeat) motif, which serves as a protein-protein interaction module found in a number of functionally different proteins (Das et al. 1998). The involvement of β-1,3-endoglucanase (spot 40) in plant defense against fungal pathogen attack has been well documented (Daugrois et al. 1990; Keen and Yoshikawa 1983; Schrőder et al. 1992). Its expression was induced in both compatible and incompatible interactions, but the increase was significantly higher and earlier in the incompatible interactions (Yi and Hwang 1996). Our study found that endoglucanase increased at a late stage of infection (12 dai) in this compatible interaction. *CHI1* (spot 8) also was highly expressed and mostly induced compared to other members of the family (such as *CHI2* and *CHI3*) in responding to ASR infection. CHI functions in flavonoids biosynthetic pathway, which is involved in the synthesis of phytoalexins and the cell wall reinforcing metabolites as part of plant defense responses (La Camera et al. 2004).

Most of the remaining sequenced proteins belong to the plant metabolism, growth and development group, and were down-regulated after rust infection except cytosolic glutamine synthetase (spot 26) and the S-adenosylmethionine synthetase 1 (spot 32). Although these proteins primarily function in plant metabolism or growth and development, there are also studies indicating their involvement in host resistance. Different isoforms of 28/31kD glycoprotein or vegetative storage protein (VSP) (spots 13, 38, and 39) were down-regulated upon rust infection in the present study. This group of proteins, which contains a conserved acidic phosphatase motif (DXDXT) and has phosphatase activity and anti-insect activity (Liu et al. 2005), has been shown to be associated with disease resistance in *Phaseolus vulgaris* against *Pseudomonas syringae* (Jakobek and Lindgren 2002) and in barley against powdery mildew (Beβer et al. 2000). S-adenosylmethionine synthetase 1 (spot 32) is an enzyme catalyzing the formation of S-adenosylmethionine, which is a major methyl-group donor and an intermediate in

the biosynthesis of the phytohormone ethylene. It has been reported that S-adenosylmethionine synthetase 1 is involved in host pathogen interaction in parsley against an elicitor from *Phytophthora megasperma* f. sp. *glycinea* (Kawalleck et al. 1992), and in barley upon powdery mildew infection (Caldo et al. 2004). In our study, a soybean cytosolic glutamine synthetase 1 (GS 1, spot 26), one of the isoform of GS that are highly active in senescent leaves, was also highly up-regulated in rust infected soybean leaves 12 dai, corresponding to defoliation caused by rust infection, indicating an increase in ammonium reassimilation and nitrogen remetabolization (Habash et al. 2001). Cytosolic GS1 might also be involved in plant defense, since *GS1* gene was induced in bean by *Colletotrichum lindemuthianum* infection (Tavernier et al. 2007).

Spots 14 (α-soluble N-ethylmalmeimide sensitive attachment protein, SNAP) and 37 (cyclophilin or peptidyl prolyl cis trans isomerase) belong to the third group (protein modification or transport) and were up-regulated upon rust infection. SNAP plays a critical role for stable binding of n-ethylmaleimide-sensitive factor (NSF) and NSF's ATPase activity during vesicular trafficking (Subramaniam et al. 1997; Whiteheart and Matveeva 2004). Its up-regulation in rust infected leaves compared to control leaves may be the result of responding to increased protein trafficking inside infected soybean leaves. The expression of cyclophilin (spot 37), which functions in catalyzing peptidyl-prolyl isomerisation to increase protein stability and is consequently involved in protein folding (Wang and Heitman 2005), was also increased during the wheat and *Puccinia triticina* (leaf rust) susceptible interaction (Rampitsch et al. 2006).

The fourth group (rust protein) includes spot 19 since its peptide sequences matched to a 3,5'-cyclic nucleotide phosphodiesterase from *Trichomonas vaginalis.* This enzyme catalyzes the hydrolysis of cyclic AMP to form adenosine 5'-phosphate and was required for hyphal development in *Candida albicans* (Jung and Stateva 2003). It also may play a role in

morphological conversion and in pathogenesis in *Magnaporthe grisea* and *Ustilago maydis* (Choi and Dean 1997; Kruger et al. 2000). Therefore, it is speculated that this protein may play a role in the infection of soybean.

3.4.3 CHI1 and PR10 Expression at Transcript and Protein Levels

In order to understand how soybean and *P. pachyrhizi* interact at the molecular level, and to develop new strategies to control soybean rust disease in the future, the transcript levels of these two proteins during the time-course of rust infection also were examined using real-time PCR. The expression of both genes showed a clear fluctuation: first induction was observed at 10 hai followed by a down-regulation at 1 dai and another induction around 2 dai (or from 6 to 8 dai). Similar biphasic induction has also been reported in recent microarray studies of soybean genes upon rust inoculation (Panthee et al. 2007; van de Mortel et al. 2007).

We further compared *CHI* transcript level with its corresponding protein and enzyme activity levels during the time-course of rust infection. *CHI* expression pattern found in this study agreed with the result of van de Mortel and associates (2007) who reported that *CHI* expression was biphasic in response to ASR infection. Among the three *CHIs* we examined, *CHI1* had the highest abundance and showed the most induction after rust infection. *CHI2* expression did not respond to the ASR infection significantly except at only one time point, 6 dai. Although *CHI3* showed a higher relative level of induction comparable to *CHI1* at a later stage of rust infection, the transcript abundance of *CHI1* was always higher than *CHI2* and *CHI3* in non-inoculated and inoculated leaves (Fig. 3.7). Although their amino acid sequence homology was low and gene expression patterns were different from each other in this study, they bind to the same substrate and generate the same product called naringenin (chalcone) which is the precursor of the phytoalexin (Shimada et al. 2003). The overall *CHI1* expression pattern is different from CHI1 protein level, which was significantly increased only 6 dai.

The corresponding enzyme activity pattern after rust infection follows the pattern of CHI1 at protein level. Similar results were reported in bean (*Phaseolus vulgaris*) after fungal elicitor treatment (Cramer et al. 1985).

PR10 gene expression level was also studied. Overall, *PR10* transcript was significantly induced at two time points, 10 hai and 6 dai in infected leaves, while protein expression was significantly induced as early as 10 hai and continued to increase throughout the time-course. Therefore, the gene expression pattern was different from protein expression pattern during the time-course. The overexpressed PR10 did not appear to have ribonuclease activity (unpublished data, Park and Chen, 2010), although the deduced amino acid sequence of the cloned PR10 gene contains a conserved P-loop motif, which has been considered required for the ribonuclease activity reported in some other PR10 proteins (Bantignies et al. 2000; Kim et al. 2008; Saraste et al. 1990; Wu et al. 2003). This lack of correlation between the levels of a transcript and its corresponding protein has been reported in earlier studies (Gygi et al. 1999; Lee et al. 2004), indicating that PR10 expression might be translationally or post-translationally regulated (Lee et al. 2004).

3.4.4 Possible Function of PR10 and CHI in Soybean Response to Rust infection

CHI is involved in the synthesis of flavonoid pigments, anthocynin and isoflavonoid phytoalexin for pollination, seed dispersal, protection of DNA from UV damage, and for plant defense (Bednar and Hadcock 1988; Dixon et al. 1983). In this study, we found that *CHI1* was highly induced in soybean leaves after rust infection. *CHI* also was induced by soybean rust infection in earlier microarray studies (Panthee et al. 2007; van de Mortel et al. 2007). Further investigation found its enzyme activity showed significant increase as early as 10 hai, although its protein level increased slowly after rust infection and was not significant until 6 dai, indicating it may play a critical role in host response to rust infection. In addition, many studies

found that *CHI* was induced by pathogen invasion. It has been reported that *CHI* increased early in the incompatible interaction of *Phaseolus vulgaris* L. and *Colletotrichum lindemuthianum* (casual agent of anthracnose) prior to phytoalexin accumulation, in contrast to its expression being delayed in a compatible interaction (Cramer et al. 1985; Mehdy and Lamb 1987).

PR10 has been reported as a defense protein in soybean (Chou et al. 2004), rice (Kim et al. 2004), pepper (Park et al. 2004), peanut (Chadha and Das 2006), and maize (Chen et al. 2006). Its transcript also was reportedly induced during rust infection of soybean (van de Mortel et al. 2007). In the present study, we demonstrated that PR10 increased rapidly at both transcript and protein levels and is the most induced protein after rust inoculation. Another interesting aspect of this PR10 is that the same *PR10* showed a similar gene expression pattern in response to a treatment with *Phytophthora sojae* cell wall elicitor, which was highly induced at 8 hai (Graham et al. 2003). This early response of the same *PR10* to infections by different pathogens suggests that PR10 is part of a host's initial but rather nonspecific response to pathogen infection. Recently, some studies demonstrated cytokinin binding activity of PR10 (Zubini et al. 2009) and reported structural similarity to a regulatory components of ABA receptor (RCAR) (Ma et al. 2009) and pyrabactin resistance 1 (PYR1) (Park et al. 2009). However, the precise function of PR10 needs to be further investigated.

In summary, differentially expressed soybean leaf proteins after *P. pachyrhizi* infection during a compatible interaction were identified and sequenced. Most of the induced proteins appear to have a role in plant defense, stress, protein modification or transport, whereas proteins involved plant growth and development were suppressed after rust infection. The changes for some of the proteins can be detected as early as 10 hai whereas other proteins were induced only at a late stage of rust infection. Two such proteins, PR10 and CHI, were further characterized, and the data suggested that both play important roles in host response to rust infection. Further

studies involving virus induced gene silencing to reduce the expression of these two proteins will be necessary to provide more definite answers as to their functions in soybean resistance to rust infection.

CHAPTER 4
EVALUATION OF SOYBEAN ACCESSIONS FOR THEIR RESPONSE TO A LOUISIANA ISOLATE OF *PHAKOPSORA PACHYRHIZI* AND PROTEOMIC ANALYSIS OF RESISTANT AND SUSCEPTIBLE LINES

4.1 Introduction

Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi*, was first reported in Japan in 1902 and later in China. The disease spread through most of the soybean producing areas in South America, including Brazil and Paraguay, by 2001 (Yorinori et al. 2005). Soybean rust is now a major emerging disease in the continental United States since its discovery in late 2004 in Louisiana (LA). *P. pachyrhizi* infection can cause quick defoliation and severe yield losses ranging from 50 to 80% (Yang et al. 1991). According to models of disease epidemiology (Yang et al. 1991) and studies of soybean rust spore viability under southern U.S. winter conditions (Jurick II et al. 2008; Park et al. 2008), soybean rust is expected to establish in the south and spread gradually to the north and will pose a serious threat to U.S. soybean production in the future.

All U.S. commercial soybean cultivars are susceptible to the fungus, and the only method to control this disease is through timely and costly fungicide applications. Therefore, there is an urgent need to develop varieties that are resistant or tolerant to ASR to reduce its potential to cause yield losses in the U.S. In an effort to develop soybean varities resistant to ASR, soybean germplasm collections have been screened. Soybean accessions resistant to *P. pachyrhizi* isolates collected from different countries, such as India, Taiwan, Nigeria, Paraguay, Vietnam, and U.S., were identified (Miles et al. 2008; Pham et al. 2009). Three reaction types of soybean accessions after inoculation with *P. pachyrhizi* have been described, including immune reaction, reddish-brown reaction with limited sporulation and fungal growth, and tan reaction

with fully sporulating uredinia (Miles et al. 2006; Bonde et al. 2006). Four single dominant genes (*Rpp1, Rpp2, Rpp3,* and *Rpp4*) confering resistance to specific isolates of *P. pachyrhizi* have been reported (Hidayat and Somaatmadja 1977; Bromfield and Hartwig 1980; Hartwig 1986). However, it has been reported that the effectiveness of resistance can be quickly overcome by virulent ASR isolates collected from different places (Hartman et al. 2005). For example, soybean accession PI200492 containing the *Rpp1* gene showed immune response to an Indian isolate, but it produced a tan or RB reaction to the other ASR isolates (Pham et al. 2009). Soybean rust isolates collected from different regions in the U.S. also produced different types of reactions on the same soybean accession (Pham et al. 2009). Thus, developing broad spectrum durable ASR resistance has not been successful.

Recently, microarray studies have been conducted to understand how soybean rust infects the host and how the host responds to pathogen attack at the molecular level using resistant lines containing the *Rpp1* or *Rpp2* resistance genes (Choi et al. 2008; van de Mortel et al. 2007). It was found that most of the rust infection induced genes were defense-related, and these genes were induced earlier and with higher intensity in a resistant line. However, a microarray analysis is limited to gene expression at the transcript level, which may have a poor correlation with its expression at protein level (Gygi et al. 1999). A proteomic approach has been successfully used to examine host-pathogen interactions in studies between bean and *Uromyces appendiculatus* (Lee et al. 2009), barrel-clover and *Orobanche crenata* (Castillejo et al. 2009), wheat and *Puccinia triticina* (Rampitsch et al. 2006), rice and *Magnaporthe grisea* (Kim et al. 2004), and between maize and *Aspergillus flavus* (Chen et al. 2004). Kim et al. (2004) found several pathogen-responsive proteins induced earlier and stronger in an incompatible interaction than a compatible one in rice during the rice blast fungus infection, including ß-1,3-glucanases, peroxidase, and pathogenesis-related 10. They concluded that a timely expression of

defense-related proteins was important for conferring host resistance. Proteomic comparison between *Medicago truncatula* and *Orobanche crenata* identified differentially expressed defense and stress-related proteins between early-resistant and late-resistant genotypes and after pathogen inoculation (Castillejo et al. 2009). They also suggested the existence of a functional genetic defense mechanism during early stages of infection that differed between the two genotypes. A proteomic approach also has been used to study soybean and ASR interaction in our laboratory (Park et al. 2010). Differentially expressed proteins belonging to plant defense, protein modification, and development were identified in this compatible interaction. Moreover, a follow-up time-course study showed that proteins relating to defense mechanisms were rapidly induced responded fast with higher levels after ASR infection.

In this study, we screened 12 accessions previously identified as resistant to rust isolates from other regions to determine their response upon inoculation with a LA rust isolate, and compared leaf protein profile differences between two resistant and two susceptible soybean lines with or without inoculation. Protein profiles of two resistant lines were compared with each of two susceptible accessions separately. Common spots only induced by ASR infection in both resistant lines after separate comparisons to each of the susceptible accessions were selected for sequencing and identification to attempt to elucidate the molecular basis of resistance of soybean to Asian soybean rust disease.

4.2 Materials Methods

4.2.1 Plant Material

Twelve soybean accessions, PI200492, PI230970, PI462312, PI417089A, PI518671, PI398998, PI437323, PI506863, PI398288, PI587905, PI567351B, and PI567104B, were screened in this study using a LA ASR isolate. PI548631 (Williams) and a commercial cultivar,

93M60 (Pioneer, Johnston, IA) were used as susceptible plant genotype in this study. Seeds were kindly provided by the Plant Genetic Resources Unit, USDA-ARS, Urbana, IL. Soybean plants were grown in 20-cm-diameter plastic pots (four plants per pot) in the greenhouse.

4.2.2 Inoculation of Detached Soybean Leaves and Plants in the Greenhouse with *P. pachyrhizi*

Asian soybean rust (*Phakopsora pachyrhizi*) urediniospores used for this study were collected from naturally infected soybean leaves (10/20/2007) at the Ben Hur Experiment Station, of Louisiana State University located in Baton Rouge, LA and kept at -80 °C. The frozen spores were resuspended in deionized water containing 0.01% Tween 20 after warming up to room temperature.

For the detached leaf assay, four pots were prepared for each accession, and the fourth to sixth trifoliate leaves were collected at R1 to R2 growth stages for inoculation with a rust spore suspension. Spore concentration was determined using a hemocytometer and adjusted to 2500 spores/ml. Two hundred microliters of inoculum containing, 500 spores were applied evenly to the adaxial surface of each of the detached soybean leaves that had been washed three times with deionized water and air-dried. Inoculated leaves were placed adaxial surface up on filter paper soaked with sterile, distilled water in 9-cm-diameter Petri dishes. The inoculated leaves were incubated under the following conditions: 26 ± 0.5 °C, 16 h day (about 50 µE $S^{-1}m^{-2}$) and 20 ± 0.5 °C, 8 h night. High moisture inside Petri dishes was maintained by adding 3 ml of deionized water every 4 days. Total numbers of lesions were counted at 14 days after infection. This experiment was conducted twice with two replicates during 2007 and 2008 using same spors. Each replicate consisted of 24 detached soybean leaves: half of them inoculated with rust urediniospores and the other half with water containing 0.01% Tween 20. The data from two

repeated experiments were combined and compare mean number of lesions per leaf between accessions.

For greenhouse accession evaluation, 39 pots, 3 pots for each soybean accessions, at R1 or R2 grown stage were inoculated by spraying 200 ml of a urediniospore suspension at 5 x 10^4 spores per ml and maintained at 25 °C in the dark with 100% humidity for 2 days before returning to greenhouse. One pot containing four plants for each accession was inoculated with 100 ml of water containing 0.01% Tween 20 as a control. Total number of lesions was counted at 14 days after infection. This greenhouse evaluation was conducted twice concurrent with the detached leaf assay.

4.2.3 Sample Preparation of Two Resistant, PI417089A and PI567104B, and Two Susceptible, PI548631 and 93M60 for Proteomic Analysis.

Two resistant, PI417089A and PI567104B, and two susceptible, PI549631 and 93M60 were selected for comparison to identify commonly up-regulated proteins in both infected resistant accessions after comparison to infected susceptible lines with *P. pachyrhizi*. One hundred sixty plants in 40 pots at R1 or R2 grown stage of two resistant and two susceptible accessions were inoculated by spraying 200 ml of a urediniospore suspension with 5 x 10^4 urediniospores per ml and maintained at 25 °C in the dark with 100% humidity for 2 days before returning to greenhouse. Another 160 plants in 40 pots were sprayed with 200 ml of sterile, distilled water containing 0.01% Tween 20 for each accession to serve as controls. The fifth to sixth trifoliate leaves from non-inoculated and inoculated plants were harvested at each time point, 0 h, 6 h, 10 h, 1, 2, 4, 6, and 12 d, after inoculation and frozen immediately using liquid nitrogen. Soybean leaf tissue was ground in liquid nitrogen using a mortar and pestle. Protein was extracted using phenol followed by methanolic ammonium acetate precipitation according to Hurkman and Tanaka (1986). Pellets were washed twice with ice-cold 0.1 M ammonium acetate

in 100% methanol with 10 mM DTT and twice with 80% acetone with 10 mM DTT. Washed pellet was dried and stored at -30 °C. This experiment was conducted twice and each sample was run in triplicate.

4.2.4 Gel Electrophoresis, Staining, and Analysis

Protein pellets were solubilized in lysis buffer (8 M urea, 4% CHAPS, 40 mM DTT, and 2% wt/vol IPG buffer). The mixture was centrifuged for 10 min at 14,000 rpm at 20 °C. Supernatant was transferred to a new tube and protein concentration was measured using the Bradford method (Bradford, 1976). Immobiline DryStrip (pH3.0 to 10.0 NL, 24 cm; GE healthcare Biosciences, Pittsburgh, PA) were rehydrated overnight at room temperature in 350 µl of rehydration solution (8 M urea, 2% CHAPS, 20 mM DTT, bromophenol, and 0.5% IPG buffer) with 150 µg of protein for silver staining (analytical) and in 450 µl of rehydration solution with 700 µg protein for Coommasie blue staining (preparative). The first and second dimensions of gel electrophoresis were performed essentially as described in Chapter 3 in this thesis. Protein spots in analytical gels were stained with Silver Stain Kit (Bio-Rad) and preparative gels were stained with 0.125% Coomassie Brillant Blue R-250 (Chen et al. 2004). All stained gels were scanned using a PowerLook II scanner (UMAX data systems, Taiwan) and analyzed using the Progenesis software (Nonlinear USA Inc, Durham, NC) to identify differentially expressed protein spots. Protein profiles from leaf collected at 1 dai were were analized to find common spots which showed over 1.5-fold difference in both infected resistant accessions compared to the controls with the $p \leq 0.05$ after Student *t*-test using SAS (version 9.1; SAS Institute, Cary, NC) Among those spots, 16 spots were selected for sequencing. Gel comparison strategies were illustrated in Figure 4.6.

4.2.5 Peptide Sequencing

Protein spots were recovered from three Coomassie-stained prep 2-D gels. These spots were subjected to an in-gel trypsin digestion (Shevchenko et al. 1996) and sequenced using liquid chromatography and tandem mass spectrometry (LC-MS/MS) at the Pennington Biomedical Center (Baton Rouge, LA).

4.2.6 Database Sequence Homology Analysis

Resulting peptide sequences were compared using BLAST (Altschul et al. 1997) to known protein in the databases at the National Center for Biotechnology Information (NCBI) and SWISS-Prot to determine their identities or homologies.

4.2.7 Fungal DNA Extraction and Absolute Quantification Using Real-time PCR

Genomic DNA was extracted from infected leaves collected at each time-point, 0 h, 6 h, 10 h, 1, 2, 4, 6, and 12 d using DNeasy plant maxi kit (Qiagen). Absolute quantification using real-time PCR was conducted with specific primers (forward: Ppm1 5′-GCAGAATTCAGTGAATCATCAAG-3′and reverse: Ppa2 5′-GCAACACTCAAAATCCAACAAT-3′) and probes (5′-FAM-CCAAAAGGTACACCTGTTTGAGTGTCA-TAMRA-3′) (Frederick et al. 2002) ampifying internal transcribed spacer (ITS) region *Phakopsora pachyrhizi* using Taqman 2x Universal PCR Master Mix (Applied Biosystems) in 25 µl reaction volume with 10 ng of genomic DNA, 12.5 µl of 2x universal PCR mixture, and 5 µM of each primer. ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used for real-time PCR under standard conditions. Amount of fungal DNA was calculated using a standard curve. Fungal DNA was extracted from total pure 4 x 10^6 spores using DNeasy plant maxi kit (Qiagen) to generate a fungal DNA standard curve. Amount of fungal DNA extracted from 4 x 10^6 spores was 25 ng/µl. Ten-fold serial dilutions were prepared from 25ng/µl to 0.25pg/ µl. One µl of template was used for real-

time PCR using the same conditions described above. PCR efficiency was 98%, R^2 was 0.99, and the slope was -3.35 for the standard curve.

4.3 Results

4.3.1 Screening Soybean Accessions to Identify Resistant to a LA Soybean Rust Isolate Using a Detached-Leaf Assay and Greenhouse Evaluation

A high number of tan lesions with many sporulating pustles were detected in both the detached leaf assay and greenhouse inoculation on accessions, PI548631 (William), PI 518671 (William 82), PI398998, PI437323, PI506863, PI398288, PI587905, and PI567351B (Fig. 4.1, Fig. 4.2) indicating a susceptible reaction. PI230970 containing *Rpp2*, which is an ASR resistant gene, showed a reddish-brown (RB) reaction (Fig 4.2) with a few sporulating pustules (Fig. 4.1) and fewer number of urediniospores in both the detached leaf assay and greenhouse inoculation. PI462312 containing *Rpp3* showed an RB reaction with few sporulating pustles (Fig. 4.1) and urediniospores only in the greenhouse inoculation. It showed immune reaction in the detached leaf assay. PI200492 (also containing *Rpp2*), PI417089A and PI567104B showed an immune reaction (Fig 4.2) in both the detached leaf assay and greenhouse inoculation. Commercial cultivar, 93M60, produced a tan reaction with a similar number of lesions between the detached leaf assay and greenhouse inoculation (Fig. 4.1, Fig 4.2).

More lesions were observed on the greenhouse inoculated leaves, which contained upto10 times higher numbers of soybean rust urediniospores per leaf than the inoculated detached leaves of the same accession. The detached leaf assay didn't showed significant variation in number of lesions among the accessions resulting tan or reddish-brown ractions, whereas the greenhouse inoculation method resulted in significant variation among the susceptible accessions. Although the number of lesions produced on leaves of same accession varied between the two methods, the reaction type was same for all accessions with both

methods (Fig 4.2). Evaluation results in this study were compared to results from studies conducted by others (Table 4.1). The types of reaction for most soybean accessions to the study LA ASR isolate were similar to what was reported in previous studies except for PI417089A, PI398998, PI398288, and PI567104B. Tan and RB reactions were detected on leaves of PI417089A after infection with Vietnam and Nigeria isolates (Adeleke et al. 2006; Vuong et al. 2006), respectively, whereas this accession showed immune reaction to the LA isolate. PI398998 showed an RB reaction after infection with a Vietnam isolate, but it responded to the LA isolate infection as a tan reaction (Table 4.1). PI398288 and PI567104B showed immune and RB reactions with sporulation to a Paraguay isolate, while they exhibited an RB reaction with sporulation and an immune reaction to the LA ASR isolate, respectively.

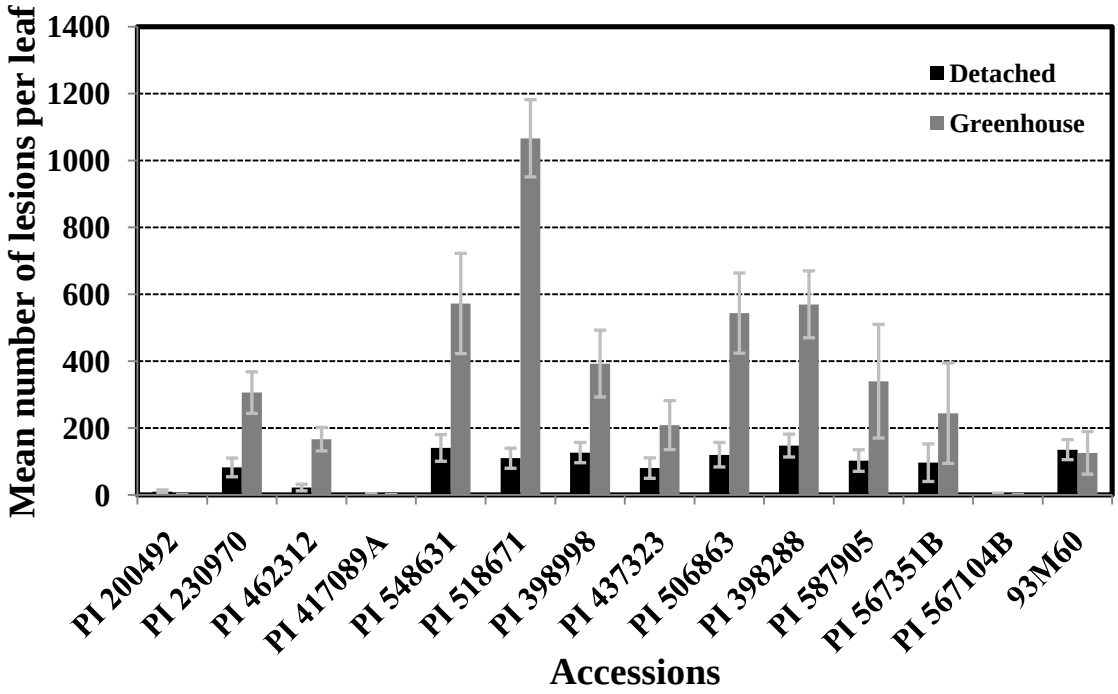


Figure 4.1. Comparison of mean number of lesions per leaf among different accessions using detached leaf assay and greenhouse inoculation with a LA ASR soybean rust isolate. The number of lesions was counted at 14 days after inoculation. Bars represent standard deviation.

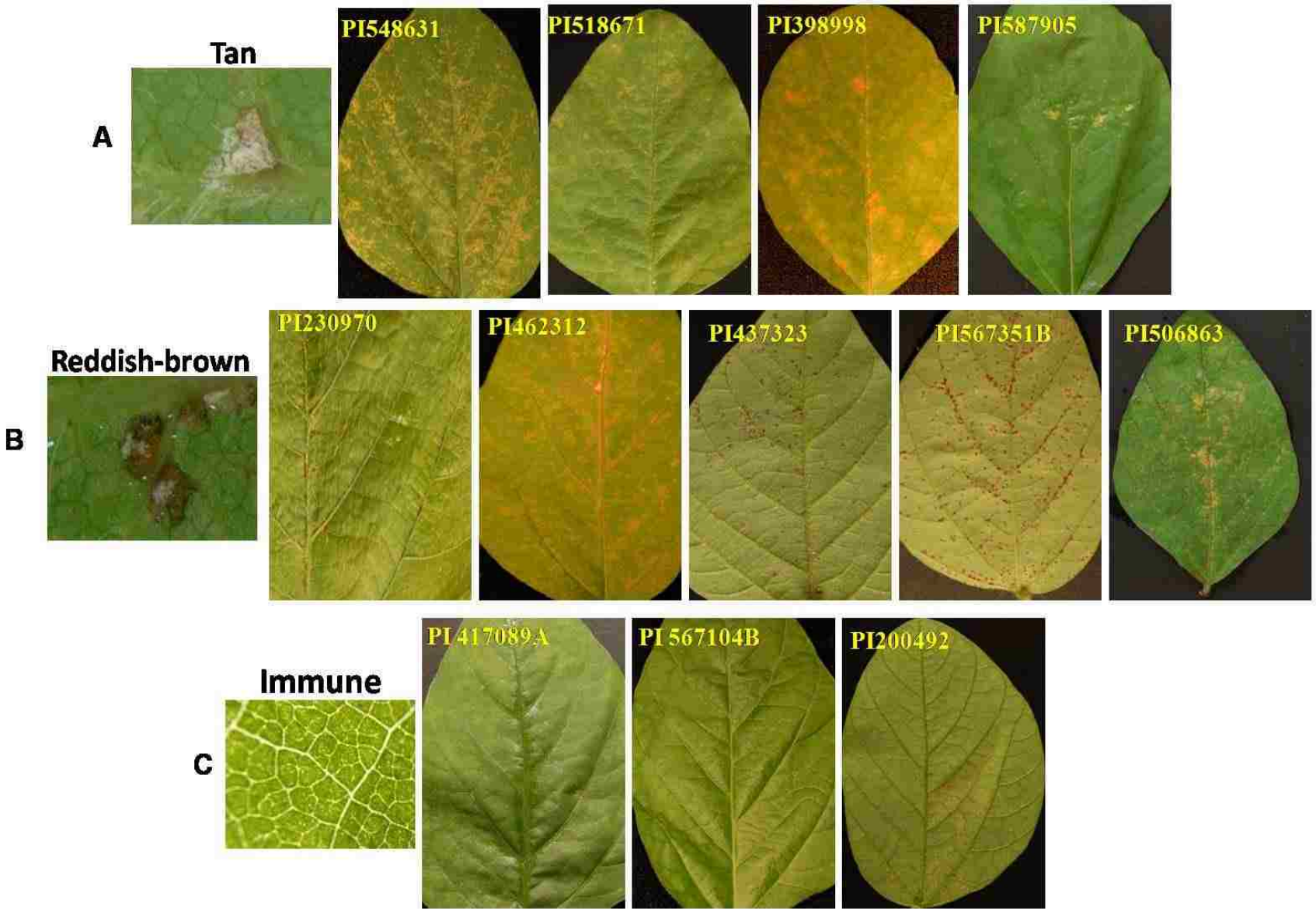


Figure 4.2. Different types of host reactions 14 days after LA soybean rust isolate infection. A, Immune reaction; B, reddish-brown reaction; C, tan reaction.

Table 4.1. Comparison of reaction types of various accessions between this study and studies conducted by others.

Accession	Maturity group	Reaction type (By others)	Named single gene	Reaction type using a LA ASR isolate
PI200492	VII	RB with sporulation to LA isolate, Pham et al. 2009	*Rpp1*	Immune or RB
PI230970	VII	RB with sporulation to LA isolate, Pham et al. 2009	*Rpp2*	RB with sporulation
PI462312	VIII	RB with sporulation to LA isolate, Pham et al. 2009	*Rpp3*	RB with sporulation
PI417089A	IX	Tan to Vietnam isolate, Vuong et al. 2005 RB to Nigeria isolate, Adeleke et al. 2006		Immune
PI548631	III	Not tested	Williams	Tan
PI518671	III	Tan to LA isolate, Pham et al. 2009	Williams 82	Tan
PI398998	VI	RB to Vietnam isolate, Vuong et al. 2005		Tan
PI437323	III	RB to LA isolate, Pham et al. 2009		RB
PI567351B	III	RB with sporulation to Paraguay isolate, Miles et al. 2008		RB with sporulation
PI506863	IV	RB with sporulation to Paraguay isolate, Miles et al. 2008		RB with sporulation
PI398288	V	Immune to Paraguay isolate, Miles et al. 2008		RB with sporulation
PI587905	VII	Tan to LA isolate, Pham et al. 2009		Tan
PI567104B	IX	RB with sporulation to Paraguay isolate, Miles et al. 2008		Immune

4.3.2 Comparison of *P. pachyrhizi* DNA Accumulation in Inoculated Leaves between Two Resistant and Two Susceptible Accesions

Successful fungal infection was verified by measuring the abundance of fungal DNA using real-time PCR in two resistant and two susceptible accessions. Ct value was converted to the fungal DNA amount compared to the standard curve generated by serial dilution of *P. pachyrhizi* genomic DNA. Significantly different fungal DNA accumulation was detected from 4 dai between the resistant and susuceptible lines.At 4 dai, 7.4 and 8 pg of fungal DNA per 1 ng of infecected soybean DNA were detected from susceptible accessions, PI548631 and 93M60, respectively. They were about 40 times higher than amount of fungal DNA initially used for inoculation at 0 hai, and 7 and 40 times higher than fungal DNA accumulation of the resistant lines, PI567104B and PI417089A. At 12 dai, 178 and 280 pg of fungalDNA per 1 ng of infected soybean DNA were detected from susceptible accessions, PI548631 and 93M60, respectively. They were about 900 to 1400 times higher than amount of fungal DNA initially used for inoculation at 0 hai, and about 100 and 1400 times higher than fungal DNA accumulation of the resistant lines, PI567104B and PI417089A. While the two susceptible lines had similar levels of fungal DNA accumulation, different amounts of fungal DNA were detected between the resistant accessions with PI567104B accumulating 14 times higher fungal DNA than PI417089A, which showed almost no fungal accumulation (Fig 4.3). However, PI567104B still showed an immune response to the LA ASR isolate (Fig 4.2).

4.3.3 Protein Profile Comparisons between Two Resistant and Two Susceptible Accessions at One Day after Infection

We detected slightly different amounts of fundgal DNA accumulation between two resistant and two susctible lines from 2 dai. We think proteins involved in this difference may be expressed ahead of 2 dai. This is the reason that we decided to compare protein profiles of resistant to susceptible accessions at 1 dai (Fig 4.4).

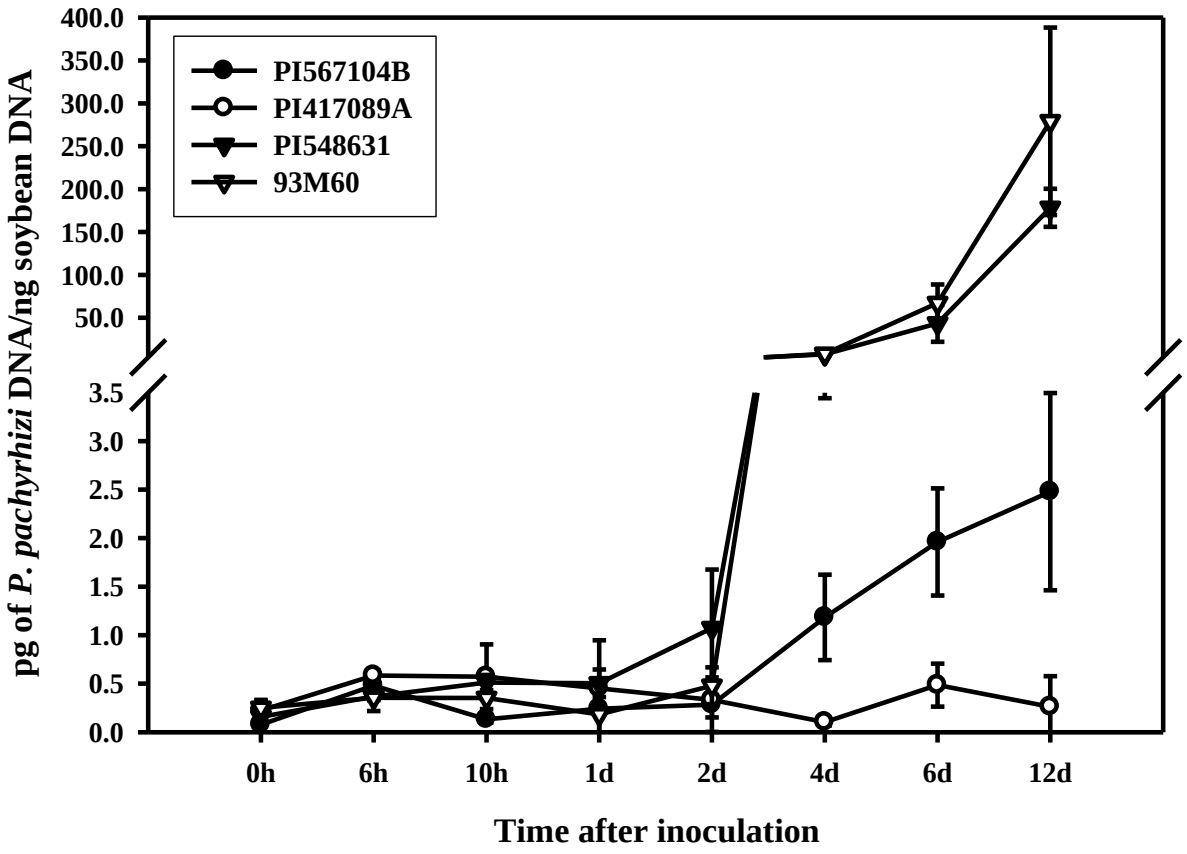
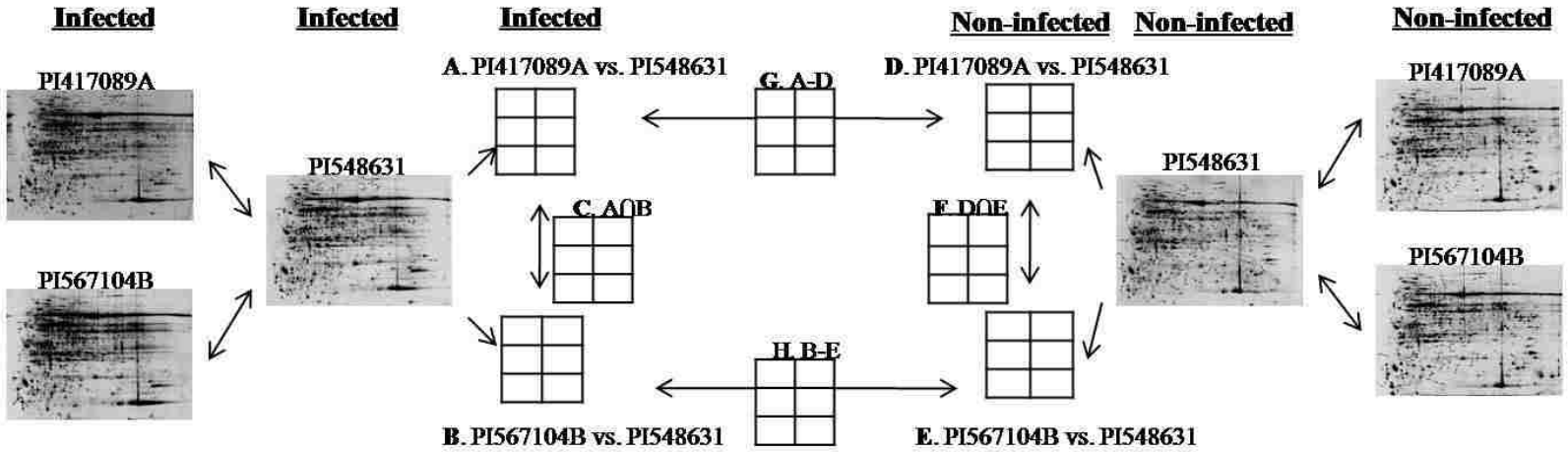


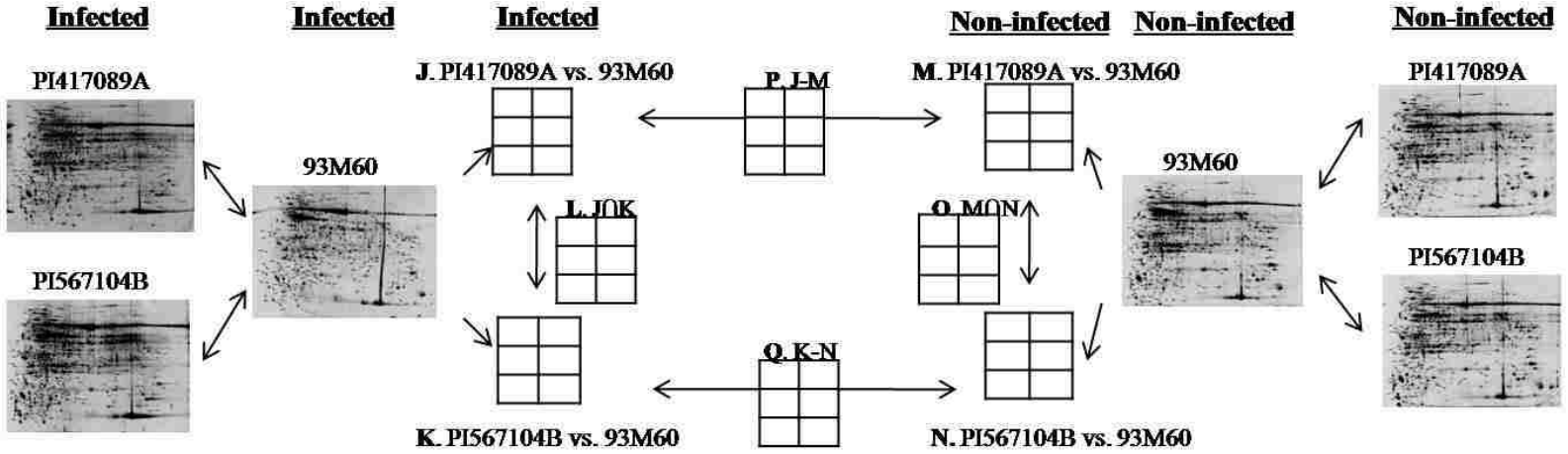
Figure 4.3. Accumulation of soybean rust DNA in infected leaves of four different accessions during the time-ourse of rust infection using quantitative real-time PCR.

Induced protein spots were found in both infected resistant accessions after separate comparisons to each susceptible accession. First, 40 spots were found up-regulated after the comparing protein profile of infected resistant line PI417089A at 1 dai to that of infected susceptible PI548631 (Table 4.2). Second, 26 proteins were found induced after comparing protein profile of infected resistant, PI567104B at 1 dai to the same infected susceptible PI548631 (Table 4.3). Among them, 18 protein spots were found commonly induced by *P. pachyrhizi* infection in both resistant accessions (Table 4.4). In order to subtract protein spots that were induced in resistant accessions compared separately to the same susceptible accessions without rust infection at 1 dai, u-pregulated proteins in non-inoculated resistant accessions also were identified. First, 30 constitutively expressed proteins were identified as up-regulated in non-infected resistant PI417089A at 1 day after water inoculation compared to that of water-inoculated susceptible PI548631 (Table 4.5). Second, 31 constitutively expressed proteins were found up-regulated in water-inoculated resistant PI567104B at 1 dai compared to that of water-inoculated susceptible PI548631 (Table 4.6). Among them, 18 of the constitutively expressed common protein spots were found up-regulated in both water-inoculated resistant accessions compared to PI548631 (Table 4.7). Thirty protein spots specifically induced by *P. pachyrhizi* infection in resistant accession (PI417089A) were identified (Table 4.8) after subtraction of constitutively expressed protein spots induced in non-infected resistant accession (PI417089A) compared one non-inoculated susceptible accession (PI548631) (Table 4.5) from upregulated protein spots by ASR infection in PI417089A (Table 4.2). Seventeen protein spots specifically induced by *P. pachyrhizi* infection in another resistant accession (PI567104B) were identified in the same manner as above (Table 4.9). Finally, eight common spots were found between spots induced by ASR in PI417089A and PI567104B (Table 4.10). All protein spots found in this comparison were marked with asterisk on the gel image (Fig 4.5).

The same comparison scheme was used to compare protein profile differences between the same two resistant lines and another susceptible soybean cultivar 93M60. First, 42 spots were found up-regulated in infected resistant PI417089A at 1 dai compared to the protein profile of infected susceptible 93M60 (Table 4.11). Second, 39 protein spots were induced in infected resistant PI567104B at 1 dai compared to the protein profile of infected susceptible 93M60 (Table 4.12). Among these spots, 22 were found commonly induced by ASRin both infected resistant accessions (Table 4.13) after comparing spots in Table 4.11 with spots in Table 4.12. In order to subtract protein spots that were induced in resistant lines compared to susceptible lines without rust infection at 1 dai, upregulated proteins in water-inoculated resistant lines also were identified. In water-inoculated resistant PI417089A, 21 constitutively expressed protein spots were found up-regulated at 1 dai compared to the protein profile of water-inoculated susceptible 93M60 (Table 4.14). In the other water-inoculated resistant, PI567104B, 32 constitutively expressed protein spots were found up-regulated compared to that of water-inoculated susceptible 93M60 (Table 4.15). Among them, 15 spots were found commonly up-regulated in both water-inoculated resistant accessions (Table 4.16). Thirty-four protein spots were specifically induced by *P.pachyrhizi* infection in resistant PI417089A after subtraction of constitutively expressed protein spots in non-infected resistant PI417089A identified by comparison to non-inoculated susceptible 93M60 (Table 4.17) from protein spots up-regulated in PI417089A (Table 4.11). Thirty protein spots specifically induced by ASR in the other resistant PI567104B were identified in the same manner as above (Table 4.18). Finally, 15 common spots were found between spots induced by ASR in PI417089A and PI567104B (Table 4.19). Positions of these spots were marked on the gel images with asterisk (Fig 4.6).



I, (G∩H); spots induced only by soybean rust infection in both infected resistant accessions (PI417089A and PI567104B) after comparison with one infected susceptible accession (PI548631).



R, (P∩Q); spots induced only by soybean rust infection in both infected resistant accessions (PI417089A and PI567104B) after comparison with one infected susceptible accession (93M60).

Figure 4.4. Schematic diagram of protein profile comparisons between resistant (PI417089A and PI567104B) and susceptible (PI548631 and 93M60) accessions at 1 day after infection in order to identify differentially expressed proteins.

Table 4.2. Identification of up-regulated protein spots and their fold changes in infected soybean leaves of resistant accession (PI417089A) compared to the protein profile of one infected susceptible (PI548631) at 1 dai. This table represents the comparison A listed in Figure 4.4.

Spot number	Fold change	Spot number	Fold change	Spot number	Fold change	Spot number	Fold change
48	2.802	483	2.397	790	1.518	1378	1.809
91	8.344	524	2.62	854	1.977	1405	2.06
186	2.869	549	1.747	927	1.733	1456	2
201	2.671	574	3.658	1103	1.702	1589	1.862
207	4.02	616	2.119	1139	1.989	1621	1.713
274	2.987	688	1.644	1238	1.958	1677	1.335
351	4.693	699	2.131	1240	2.246	1827	1.474
374	2.182	755	2.004	1249	2.234	1860	1.6
382	1.789	760	1.749	1340	2.017	1890	1.633
419	3.663	781	2.368	1352	2.063	2174	1.5

Table 4.3. Identification of up-regulated protein spots and their fold changes in infected soybean leaves of resistant accession (PI567104B) compared to the protein profile of one infected susceptible (PI548631). This table represents the comparison B listed in Figure 4.4.

Spot number	Fold change	Spot number	Fold change	Spot number	Fold change	Spot number	Fold change
91	10.418	816	1.762	1378	1.9	1677	2.048
274	1.773	929	2.778	1423	1.4	1827	1.666
380	1.783	1139	2.27	1442	1.941	1860	1.6
382	2.825	1249	1.886	1456	2	1890	1.7
483	1.528	1249.1	5.7	1589	1.805	1977	1.435
760	1.71	1340	1.035	1603	1.346	2289	1.5
781	2	1352	2.318	1621	1.586		

Table 4.4 Identification of common spots up-regulated in both resistant accessions (PI417089A and PI567104B) through the comparison of Table 4.2 and Table 4.3. This table represents the comparison C listed in Figure 4.4.

Spot number	Fold change		Spot number	Fold change	
	PI417089A	PI567104B		PI417089A	PI567104B
91	8.344	10.418	**1378**	1.809	1.9
274	2.987	1.773	**1442**	1.363	1.941
382	1.789	2.825	**1456**	2	2
483	2.397	1.528	**1589**	1.862	1.805
760	1.749	1.71	**1621**	1.713	1.586
781	2.368	2	**1677**	1.335	2.048
1139	1.989	2.27	**1827**	1.474	1.666
1249	2.234	1.886	**1860**	1.6	1.6
1340	2.017	1.035	**1890**	1.633	1.7

Table 4.5. Identification of constitutively expressed protein spots induced in non-infected soybean leaves of resistant accession (PI417089A) compared to the protein profile of one non-infected susceptible (PI548631). This table represents the comparison D listed in Figure 4.4.

Spot number	Fold change	Spot number	Fold change	Spot number	Fold change	Spot number	Fold change
46	3.389	1357	2.358	1976	1.733	2177	1.569
48	9	1368	2.336	1981	1.728	2226	1.512
82	3.275	1420	1.989	2000	1.5	2258	1.5
539	4.22	1442	1.749	2071	1.662	2472	1.354
972	1.5	1555	1.688	2072	1.662	2512	1.7
973	2.826	1897	1.762	2080	1.655	2514	2.5
1132	1.5	1909	1.523	2107	1.441		
1147	2.285	1937	1.759	2129	1.601		

Table 4.6. Identification of constitutively expressed protein spots induced in non-infected soybean leaves of resistant accession (PI417089A) compared to the protein profile of one non-infected susceptible (PI548631). This table represents the comparison E listed in Figure 4.4.

Spot number	Fold change	Spot number	Fold change	Spot number	Fold change	Spot number	Fold change
46	6.753	1132	1.6	1853	1.79	2107	1.266
48	9	1147	1.707	1897	1.568	2226	1.533
138	7	1168	1.546	1909	1.776	2469	3
443	3.776	1368	1.71	1937	1.754	2472	1.247
540	3.7	1378	1.83	1976	1.535	2514	2.3
563	1.709	1420	1.567	1981	1.663		
604	2.244	1442	2.255	2000	1.5		
972	1.772	1657	2.01	2071	1.3		
1109	2.717	1762	1.569	2073	1.51		

Table 4.7. Identification of common constitutively expressed protein spots induced in both non-infected resistant accessions (PI417089A and PI567104B) through the comparison of Table 4.5 and Table 4.6. This table represents the comparison F listed in Figure 4.4.

Spot number	Fold change		Spot number	Fold change	
	PI417089A	PI567104B		PI417089A	PI567104B
46	3.389	6.753	**1909**	1.523	1.776
48	9	9	**1937**	1.759	1.754
972	1.5	1.772	**1976**	1.733	1.535
1132	1.5	1.6	**1981**	1.728	1.663
1147	2.285	1.707	**2000**	1.655	1.5
1368	2.336	1.71	**2107**	1.441	1.266
1420	1.989	1.567	**2226**	1.512	1.533
1442	1.749	2.255	**2472**	1.354	1.247
1897	1.762	1.568	**2514**	2.5	2.3

Table 4.8. Identification of protein spots specifically induced by ASR infection in resistant accession PI417089A after subtraction of constitutively expressed protein spots (Table 4.5) from protein spots up-regulated in response to ASR in PI417089A (Table 4.2). This table represents the comparison G listed in Figure 4.4.

Spot number	Spot number	Spot number	Spot number	Spot number	Spot number
48	374	549	781	1249	1456
186	382	574	790	1340	1589
201	419	616	854	1352	1677
207	483	688	927	1378	1860
274	524	699	1103	1405	2174

Table 4.9. Identification of protein spots specifically induced by ASR infection in resistant accession PI567104B after subtraction of constitutively expressed protein spots (Table 4.6) from protein spots up-regulated in response to ASR in PI567104B (Table 4.3). This table represents the comparison H listed in Figure 4.4.

Spot number	Spot number	Spot number	Spot number
274	929	1423	1860
382	1249	1456	
483	1340	1589	
781	1352	1603	
816	1378	1677	

Table 4.10. Identification of common protein spots specifically induced by *P.pachyrhizi* between infected two resistant accessions (PI417089A and PI567104B) by comparing the results in Table 4.8 to Table 4.9. This table represents the comparison I listed in Figure 4.4.

Spot number	Fold change		Spot number	Fold change	
	PI417089A	PI567104B		PI417089A	PI567104B
274	2.987	1.773	**1378**	1.809	1.9
382	1.789	2.825	**1589**	1.862	1.805
483	2.397	1.528	**1860**	1.6	1.6
781	2.368	2	**1249**	2.233	1.886

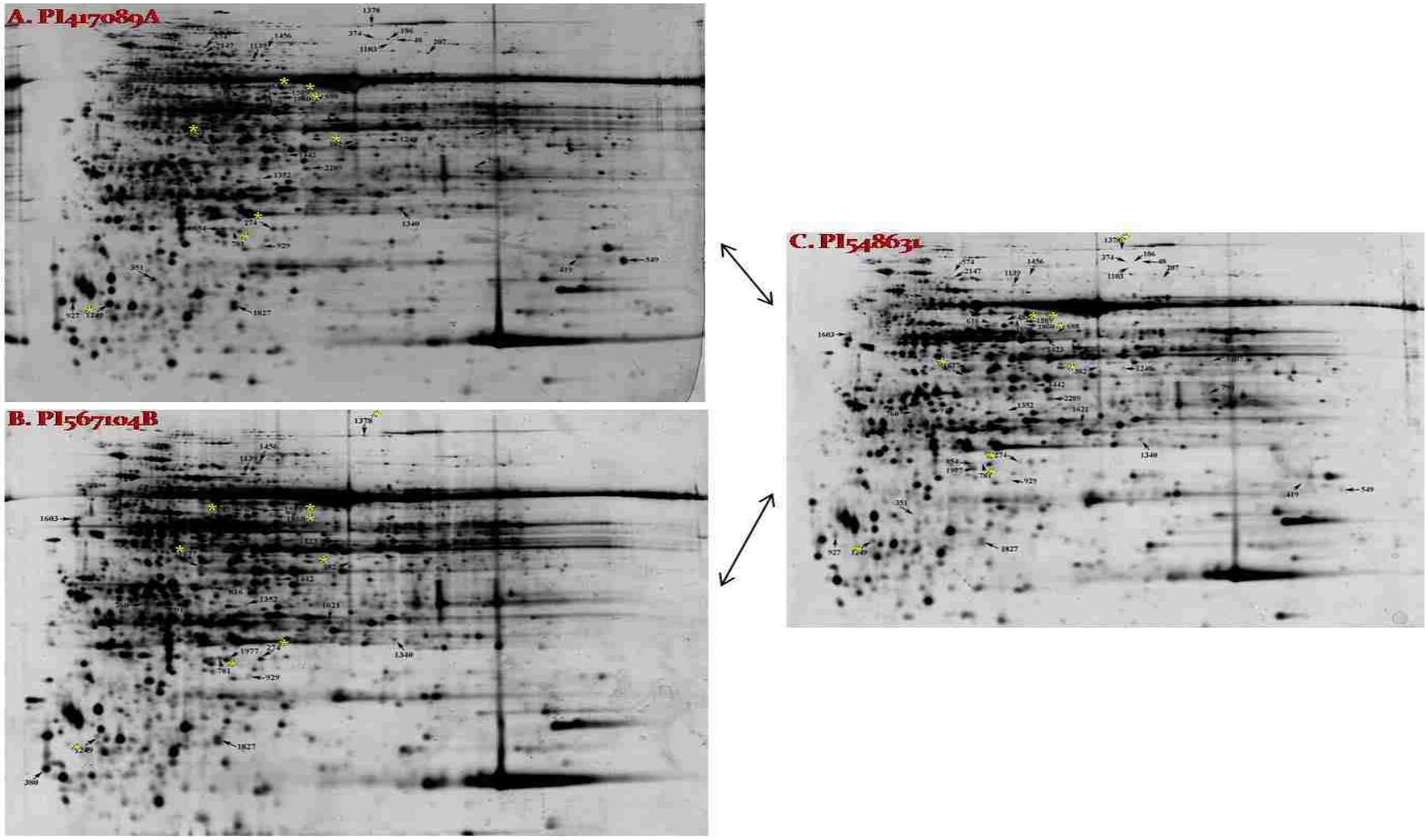


Figure 4.5. Comparison of soybean leaf 2-D protein profile differences between two resistant accessions (PI417089A and PI567104B) and one susceptible accession (PI548631) at 1 dai with *P. pachyrhizi*. The numbered spots were up-regulated in both resistant lines in response to ASR. The spots marked by asterisk were commonly induced in both infected resistant lines compared to infected susceptible one.

Table 4.11. Identification of up-regulated protein spots and their fold changes in infected soybean leaves of resistant PI417089A compared to the protein profiles of infected susceptible 93M60. This table represents the comparison J listed in Figure 4.4.

Spot number	Fold change	Spot number	Fold change	Spot number	Fold change	Spot number	Fold change
91	4.089	742	1.652	1323	1.793	1579	1.746
201	3.03	746	2.248	1327	1.465	1565	1.5
232	1.685	811	2.223	1342	1.378	1647	1.546
351	3.427	892	2.856	1340	1.5	1624	1.489
435	2.155	927	2.783	1456	1.509	1663	1.626
459	2.163	958	1.77	1458	1.723	1863	1.5
539	1.991	1035	1.898	1471	1.758	1679	1.79
605	1.987	1160	2.25	1503	1.959	1697	1.711
696	1.556	1184	1.931	1523	1.701	1707	1.278
699	2.623	1198	1.807	1542	1.506		
728	1.945	1249	2.177	1571	1.648		

Table 4.12. Identification of up-regulated protein spots and their fold changes in infected soybean leaves of resistant accession PI567104B compared to the protein profile of infected susceptible 93M60. This table represents the comparison K listed in Figure 4.4.

Spot number	Fold change	Spot number	Fold change	Spot number	Fold change	Spot number	Fold change
91	3.162	892	1.744	1249	1.598	1565	1.883
214	5.888	927	1.854	1267	1.573	1571	1.852
380	1.623	958	2.694	1278	1.748	1589	1.583
435	4.291	963	1.686	1291	2.144	1597	1.853
459	2.913	1035	1.759	1323	1.94	1640	1.673
530	1.987	1111	1.578	1327	2.136	1647	1.812
586	1.69	1130	2.4	1342	2.115	1663	1.8
728	1.618	1160	1.956	1393	2.008	1676	1.792
746	2.123	1184	1.595	1471	1.638	1249.1	4.5
851	1.909	1245	1.925	1503	1.57		

Table 4.13. Identification of common spots up-regulated in both resistant accessions (PI417089A and PI567104B) through the comparison of Table 4.11 to Table 4.12. This table represents the comparison L listed in Figure 4.4.

Spot number	Fold change		Spot number	Fold change		Spot number	Fold change	
	PI417089A	PI567104B		PI417089A	PI567104B		PI417089A	PI567104B
91	4.089	3.162	**927**	2.783	1.854	**1458**	1.723	1.424
435	2.155	4.291	**958**	1.77	2.694	**1471**	1.758	1.638
459	2.163	2.913	**1035**	1.898	1.759	**1503**	1.959	1.57
728	1.945	1.618	**1160**	2.25	1.956	**1523**	1.701	1.324
746	2.248	2.123	**1184**	1.931	1.595	**1571**	1.648	1.852
851	1.461	1.909	**1323**	1.793	1.94	**1647**	1.546	1.812
892	2.856	1.744	**1327**	1.465	2.136	**1663**	1.626	1.8
			1249	2.177	1.5			

Table 4.14. Identification of constitutively expressed protein spots induced in non-infected soybean leaves of resistant accession PI417089A compared to the protein profile of non-infected susceptible 93M60. This table represents the comparison M listed in Figure 4.4.

Spot number	Fold change	Spot number	Fold change	Spot number	Fold change	Spot number	Fold change
46	1.929	972	1.5	1498	1.773	2080	1.627
48	4.545	1132	2.595	1510	1.716	2112	1.613
82	2.831	1168	1.5	1584	2.04	2350	3
539	4.158	1378	1.5	1676	1.737		
868	1.883	1448	1.879	1909	1.776		
914	1.625	1485	1.869	2000	1.5		

Table 4.15. Identification of constitutively expressed protein spots induced in non-infected soybean leaves of resistant accession PI567104B compared to the protein profile of non-infected susceptible 93M60. This table represents the comparison N listed in Figure 4.4.

Spot number	Fold change	Spot number	Fold change	Spot number	Fold change	Spot number	Fold change
46	3.845	1109	2.242	1443	2.206	1676	1.993
48	4.707	1132	2.682	1448	1.55	1706	1.848
138	4.7	1168	2.325	1485	1.944	1826	1.695
313	2.281	1190	1.633	1498	2.022	2019	1.587
540	4.218	1191	1.661	1502	1.607	2057	1.642
868	1.996	1375	1.781	1510	1.789	2190	1.559
914	2.514	1378	1.503	1531	1.5	2350	3
972	1.758	1442	1.773	1584	2.01	2000	1.5

Table 4.16. Identification of constitutively expressed protein spots induced in both non-infected resistant accessions (PI417089A and PI567104B) through the comparison of Table 4.14 to Table 4.15. This table represents the comparison O listed in Figure 4.4.

Spot number	Fold change		Spot number	Fold change		Spot number	Fold change	
	PI417089A	PI567104B		PI417089A	PI567104B		PI417089A	567104B
46	1.929	3.845	**1168**	1.5	2.325	**1510**	1.716	1.789
48	4.545	4.707	**1378**	1.5	1.503	**1584**	2.04	2.01
868	1.883	1.996	**1448**	1.879	1.55	**1676**	1.737	1.993
914	1.625	2.514	**1485**	1.869	1.944	**2000**	1.5	1.5
1132	2.595	2.682	**1498**	1.773	2.022	**2350**	3	3

Table 4.17. Identification of protein spots specifically induced by ASR infection in one resistant accession PI417089A after subtraction of constitutively expressed protein spots (Table 4.14) from protein spots up-regulated in response to ASR in PI417089A (Table 4.11). This table represents the comparison P listed in Figure 4.4.

Spot number	Spot number	Spot number	Spot number	Spot number	Spot number	Spot number
201	728	958	1198	1393	1542	1624
232	742	1035	1249	1456	1571	1863
605	746	1130	1327	1458	1579	1813
696	811	1160	1342	1471	1565	1727
699	892	1184	1340	1503	1647	

Table 4.18. Identification of protein spots specifically induced by ASR infection in resistant accession PI567104B after subtraction of constitutively expressed protein spots (Table 4.15) from protein spots up-regulated in response to ASR in PI567104B (Table 4.12). This table represents the comparison Q listed in Figure 4.4.

Spot number	Spot number	Spot number	Spot number	Spot number	Spot number
232	958	1160	1327	1503	1589
586	963	1184	1342	1523	1597
728	1035	1249.1	1393	1557	1640
746	1111	1267	1458	1565	1647
892	1130	1291	1471	1571	1676

Table 4.19. Identification of common protein spots specifically induced by *P. pachyrhizi* infection between infected two resistant accessions (PI417089A and PI567104B) by comparing the result in Table 4.17 to Table 4.18. This table representsthe comparison R listed in Figure 4.4.

Spot number	Fold change		Spot number	Fold change		Spot number	Fold change	
	PI417089A	PI567104B		PI417089A	PI567104B		PI417089A	PI567104B
459	2.155	4.291	**1035**	1.898	1.759	**1471**	1.758	1.638
728	1.945	1.618	**1160**	2.25	1.956	**1503**	1.959	1.57
746	2.248	2.123	**1184**	1.931	1.595	**1571**	1.648	1.852
892	2.856	1.744	**1327**	1.465	2.136	**1647**	1.546	1.812
958	1.77	2.694	**1458**	1.723	1.424	**1249**	2.177	1.5

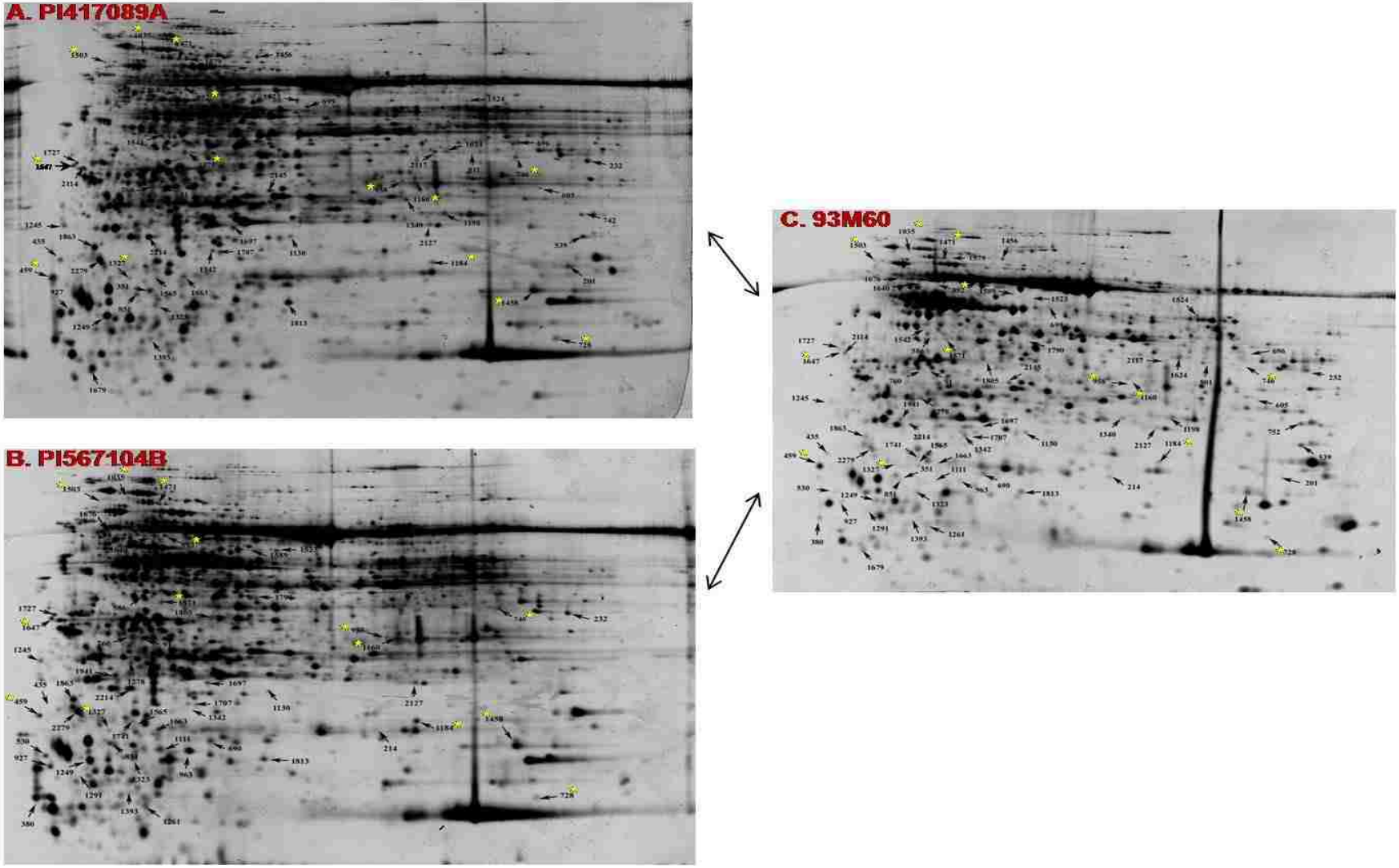


Figure 4.6. Comparison of protein profile differences between two resistant accessions (PI417089A and PI567104B) and one susceptible accession (93M60) at 1 day after with *P. pachyrhizi* inoculation. The numbered spots were up-regulated in both resistant lines in response to ASR. The spots marked by asterisk were commonly induced in both resistant lines compared to the susceptible one.

4.3.4 Sequencing and Identification of Common Protein Spots Induced by ASR Infection in Both Resistant Accessions

Six from Eight spots induced in both resistant accessions by *P. pachyrhizi* infection compared to susceptible PI548631 (Table 4.10) and 8 from 15 spots that were induced in both resistant accessions only by *P. pachyrhizi* infection compared to the susceptible cultivar, 93M60 (Table 4. 19) were recovered from Coommassie brilliant blue preparative gels (Table 4.20) for sequencing. Two additional spots, 91 and 1249.1 also were sequenced. Spot 91(spot 48 in non-infected comparison) showed the highest protein expression in both non-infected resistant accessions compared to susceptible line PI548631, which had a low level of spot 91. The expression level of this protein spot also was high in both resistant lines after infection. When the protein levels of spot 91in the two resistant lines were compared to that of the other susceptible line 93M60, its expression level was relatively low because spot 91 was highly expressed in 93M60. However, its expression level remained high after infection in both resistant accessions (Table 4.17; Table 4.18). Spot 1249.1 from infected PI 567104B and its corresponding spot 138 from non-infected PI56704B was unique and constitutively expressed at a high level, and its expression level remained high after infection.

Table 4.20. Summary of spots up-regulated over 1.5-fold in both resistant accessions in response to *P. pachyrhizi* infection that were selected for peptide sequencing.

Two resistant vs. PI548631	Two resistant vs. 93M60	Additional spots
1-274	2-728	91
1-483	2-958	1249.1
1-781	2-1160	
1-1378	2-1184	
1-1589	2-1471	
1-1860	2-1571	
	2-1647	
	2-1977	

The obtained peptide sequences and identities of the sequenced protein spots are summarized in Table 4.21. The identities of protein spots induced in both resistant accessions by infection compared to susceptible PI548631 were lactoylglutathione lyase (spot 1-274, also known as glyoxalase I, EC4.4.1.5), 1-deoxyxylulose-5-phosphate reductoisomerase (spot 1-483), ATP synthase subunit delta (spot 1-781), elongation factor 2 (spot 1-1378), phosphoglycerate kinase (spot 1-1589), and SAM-2 (S-adenosylmethionine synthetase 2) (spot 1-1860). The identities of protein spots induced in both resistant lines compared to susceptible 93M60 include FKBP type peptidyl prolyl cis trans isomerase 3 (spot 2-728), a possible membrane associated 30 kDa protein (spot 2-958), a probable protein phosphatase 2C (spot 2-1160), a nucleoside diphosphate kinase (spot 2-1184), a cell division protease ftsH homolog 2 (spot 2-1471), a ferredoxin--NADP reductase (spot 2-1571), and the DNA repair protein RAD23 (spot 2-1647). Spot 1249.1 uniquely present in infected PI567104B was identified as SAM22 (also known as PR10 protein). Spot 1977, down-regulated in PI567104B, was identified as a thylakoid lumenal 19 kDa protein. Spot 91 was identified as an oxygen evolving enhancer protein 1. The functions of these spots will be discussed in discussion section.

4.3.5 Comparison of PR10 Expression between Two Resistant and Two Susceptible Accessions during the Time-course of Rust Infection

In the previous chapter, we found that PR10 protein was induced earlier and more rapidly in response to ASR infection than any other protein in a compatible interaction. In considering using PR10 as a marker protein for initiating defense mechanism, we examined the time and level of PR10 expression in two resistant and two susceptible accessions during a time-course (Fig 4.7). Earliest PR10 protein expression was detected in PI417089A resistant accession at 6 h and 10 h after infection (hai) compared to the susceptible accessions.

Table 4.21. Identification of protein spots up-regulated in soybean leaves of two different resistant accessions inoculated with *Phakopsora pachyrhizi* through peptide sequencing using tandem mass spectrometry (MS/MS).

Spot	Top hit	Organism	GenBank	pI	MW (kDa)		Peptide sequences
1-274	Lactoylglutathione lyase	*G. max*	Q9ZS21	5.5	21	3e-13	KESPSNNPGLHTTPDEATKG KGYIMQQTMFRI KVSLDFYSRV RFQNLGVEFVKK
1-483	1-deoxyxylulose-5-phosphate reductoisomerase	*O. sativa*	NP_001041780	5.7	51	3e-07	KAILAALEAGKD KISYLDIFKV RNESLIDELKE RVILTASGGAFRD KAVEMFIDEKI KVVELTCDAHQN KITIDSATLFNKG RIYCSEVTWPRL RAGGTMTGVLSAANEKA RLPILYTLSWPERI KETLIAGGPFVLPLAKKH
1-781	ATP synthase subunit delta', mitochondrial	*P. sativum*	Q41000	7.7	21	7e-06	KVSPNIDPPKT TKLTVNFVLPYSSQLAAKE RIDANLVQKG LQEFTQKL NSATTDLEKR
1-1378	Elongation factor 2	*B. Vulgaris*	O232755	5.8	93	2e-04	RNMSVIAHVDHGKS KFSVSPVVRV KSDPVVSFRE KGVQYLNEIKD KEGALAEENMRG RIMGPNYVPGEKK KILSEEFGWDKE RGFVQFCYEPIKQ KEQMTPLSEFEDKL RNCDPEGPLMLYVSKM
1-1589	Phosphoglycerate kinase chloroplastic	*N. tabacum*	Q42961	8.6	51	5e-11	KFAVGTEAIAKK KYSLAPLVPRL KRPFAAIVGGSKV RLSELLGIQVVKV KLASLADLFVNDAFGTAHRA KELDYLVGAVSSPKR KGVSLLLPSDVVIADKF KLVASLPDGGVLLLENVRF
1-1860	SAM-2 (S-adenosylmethionine synthetase 2)	*A. thaliana*	NP_192094	5.6	43	2e-12	KEHVIKPVIPEKY KSIVANGLARR KNGTCPWLRPDGKT RFVIGGPHGDAGLTGRK KTAAYGHFGRE RKNGTCPWLRPDGKT RGGPHGDAGLTGRK REDPDFTWEVVKPLKW KTIFHLNPSGRF KVLVNIEQQSPDIAQGVHGHLTKR KIIIDTYGGWGAHGGGAFSGKD RVHTVLISTQHDETVTNDEIAADL KE METFLFTSESVNEGHPDKL KTNLVMVFGEITTKA KLCDQISDAVLDACLEQDPDSKV

Table 4.21 Continued

Spot	Top hit	Organism	GenBank	pI	MW (kDa)	e-value	Peptide sequences
2-728	FKBP type peptidyl prolyl cis trans isomerase 3 chloroplastic	*A. thaliana*	NP_199380	8.8	22	3e-13	KVFDSSYNRG RGKPLTFRI
2-958	Probable membrane associated 30 kDa protein chloroplastic	*P. sativum*	Q03943	9.5	35	2e-08	KSYADNASSLKA KILEQAVLEMNDDLTKM RQATAQVLASQKR KSYANAVLSSFEDPEKI
2-1160	Probable protein phosphatase 2C 10	*A. thaliana*	NP_174731	7.4	30	8.8	RVNGQLAVSRA KDDISCIVVRL KVMSNQEAVDVARK
2-1184	Nucleoside diphosphate kinase 2 chloroplastic	*S. oleracea*	Q01402	9.1	26	0.003	RGDLAVQTGRN KELAEEHYKD KLIGATDPLQAEPGTIRG
2-1471	Cell division protease ftsH homolog 2, chloroplastic	*O. sativa*	Q655S1	5.5	73	8e-16	RFLEYLDKDRV KIVEVLLEKE KEIDDSIDRI KETMSGDEFRA KGVLLVGPPGTGKT RFLEYLDKDRV KSLVAYHEVGHAICGTLTPGHDA VQKV KQDFMEVVEFLKK KAKENAPCIVFVDEIDAVGR Q RIVAGMEGTVMTDGKS RTPGFSGADLANLLNEAAILAGRR RVQLPGLSQELLQKL KTGVTFDDVAGVDEAKQ RLSDEAYEIALSQIRS KENAPCIVFVDEIDAVGRQ
2-1571	Ferredoxin--NADP reductase	I. cylindrica	P84210	7.5	40	6e-06	RLVYTNDQGEIVKG RLYSIASSAIGDFGDSKT KGIDDIMVSLAAKD
2-1647	DNA repair protein RAD23, putative	*A.thaliana*	NP_198663	4.5	40	0.13	RNSQQFQALRA
2-1977	Thylakoid lumenal 19 kDa protein chloroplastic	*A.thaliana*	P82658	7.4	24	1e-05	KEYLTFLAGFRQ KGTNGTDSEFYNPKK
91	Oxygen evolving enhancer protein 1 chloroplastic	*P. sativum*	P14226	6.2	34	7e-18	RVPFLFTIKQ KQLVASGKPDSFSGEFLVPSYRG RGASTGYDNAVALPAGGRG RDGIDYAAVTVQLPGGERV
1249.1	Stress induced protein SAM22	*G. max*	X60043	4.4	17	2e-06	KALVTDADNVIPKA KSVENVEGNGGPGTIKK

Its expression remained high until 6 dai and then almost no expression was detected at 10 dai. In both susceptible accessions, PR10 protein spots appeared at 10 hai but its expression level was much less than in that of PI417089A during early stages of infection. PR10 then increased from 4 dai and remained higher than resistant line PI417089A until 12 dai. Another resistant line, PI567104B showed a specific protein spot before inoculation. The position of this spot is unique compared to other accessions and is very close to PR10 protein spot. But sequencing result of this spot (1249.1) revealed that it is another isoform of PR10 (Table 4.21). The normal PR10 protein spot appeared at 10 hai, but its expression level was much lower than in PI417089A and the susceptible accessions. It was slowly induced until 6 dai and reduced at 10 dai.

4.4. Discussion

4.4.1 Identification of Two Resistant Soybean Accessions Using Detached-leaf Assay and Greenhouse Inoculation

Twelve accessions were screened using a LA ASR isolate. Most of the accessions were previously reported as resistant to isolates collected from other countries (Miles et al. 2008). Soybean accessions containing single gene resistance to rust also were tested using the LA isolate in this study. The number of lesions from the detached-leaf assay compared to those resulting from greenhouse inoculations was significantly lower in susceptible accessions. Similar numbers of lesions were observed in all accessions showed tan and RB reaction in the detached - leaf assays, whereas the number of lesions was variable among them in greenhouse inoculations. One possible explanation is that the same number of urediniospores was applied on the leaves of all the accessions for the detached-leaf assay. In addition, the environmental conditions were more strictly controlled for disease development in the detached-leaf assay compared to greenhouse inoculations.

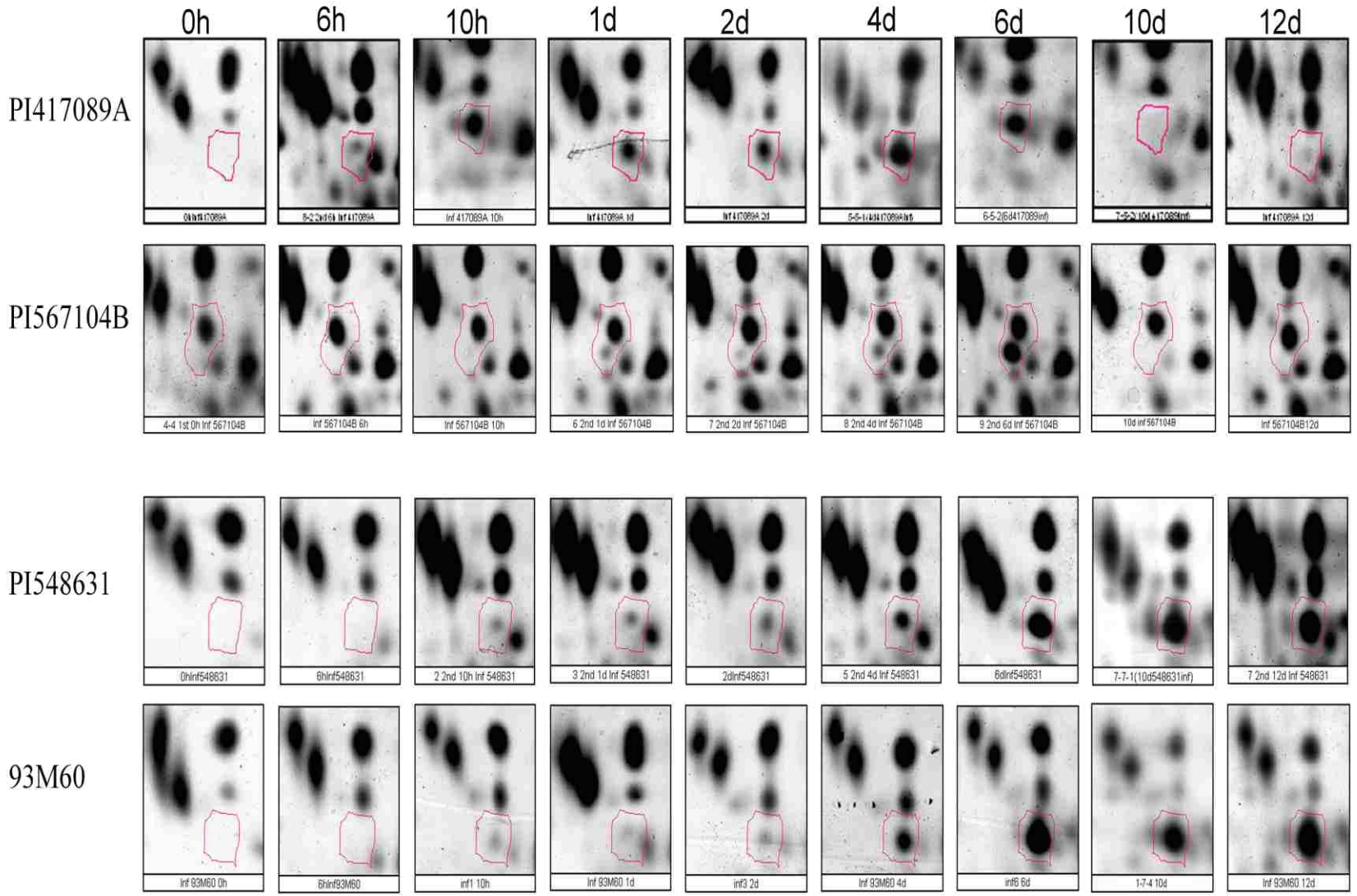


Figure 4.7. Changes of PR10 protein spot in two resistant (PI417089A and PI567104B) and two susceptible (PI548631 and 93M60) accessions during the time-course of rust infection. Red circle indicates the PR10 protein spot.

However, PI200492, PI417089A, and PI567104B produced consistent immune reactions to LA ASR isolate in both assays. In a previous study (Twizeyimana et al. 2007), field, greenhouse, and detached-leaf evaluation have been compared. They concluded that a detached-leaf assay is a very reliable and rapid method to discriminate rust resistance in soybean under laboratory conditions since all disease evaluation data from the three screening methods were well-correlated (Twizeyimana et al. 2007). Differences in the reaction of the accessions to the LA isolate were observed and compared to the reactions reported by others using different isolates in earlier studies, such as PI417089A, PI398998, PI398288, and PI567104B. These accessions have been reported to have tan or RB, immune, and RB with sporulation responses to ASR, respectively (Miles et al. 2008), but they produced a different reaction to the LA isolate in this study. Our data agree with the study by Pham et al. (2009), in which soybean accession showed a different reaction type to different rust isolates.

4.4.2 Differential Fungal DNA Accumulation between the Resistant and Susceptible Accessions as well as between the Two Resistant Soybean Accessions

A significantly higher level of soybean rust fungal DNA accumulated in both susceptible accessions compared to the two resistant accessions. Fungal DNA accumulated in PI417089A whereas a limited amount of fungal DNA accumulated in PI567104B, indicating that these two resistant accessions may have different defense mechanisms. Pham et al. (2009) arrived at a similar conclusion in their soybean accession study, in which they showed that many identified sources of resistance included in the study had reaction patterns different from the genotypes possessing the known resistance genes $Rpp_{1\text{-}4}$. They suggested that additional resistance genes are present in these genotypes (Pham et al. 2009).

When protein profiles of two resistant accessions were compared to with one susceptible, separately following infection, different unique spots were induced in each infected resistant

accessions in addition to common spots induced in both resistant accessions. These uniquely induced spots could be realted to different defense mechanisms resulting in different amount of fungal DNA accumulation. The study results suggest both different and common defense mechanism, which is conferred by the common spots induced after infection, they also have their own unique defense mechanisms, which determines their degree of resistance.The unique spots up-regulated in each infected resistant accession should be sequenced and characterized in the future

4.4.3 Differential Protein Expressions in Two Resistant Accessions Compared to Two Susceptible Accessions With and Without ASR Infection

Some of the induced protein spots found in infected resistant accessions were constitutively expressed at high levels in the same accession before inoculation. These pre-existing proteins were probably involved in a basal defense mechanism associated with MAMP (microbe-associated molecular patterns) recognition (Creelman and Mullet et al. 1995; van Loon et al. 2006). In this study, an attempt was made to identify spots that were commonly induced only in both resistant accessions (after subtracting highly expressed constitutive protein spots), that might be involved in a inducible basal host defense mechanism or the repaire of a weak basal defense (Lee et al. 2009). We also found a few of the spots, 1249 and 1340, that were up-regulated at 1 dai in resistant accessions matched to the identified spots discussed in the previous chapter identifying differentially expressed proteins during the compatible interaction between soybean and soybean rust interactions based on their physical locations in 2-D protein profiles. Spot 1249, which was up-regulated in both resistant lines only after ASR infection matched to PR10, and spot 1340, which was only induced in one resistant PI417089A after ASR infection, was matched to the previously identified CHI1. In this study, time-course experiment of PR10 showed that it was up-regulated from 6 hai and CHI1 induction was detected at 1 dai in resistant

which is 5 days earlier than the expression found in compatible interactions from previous study. It implies that PR10 and CHI1 protein may be involved in the basal defense mechanism in resistant accessions.

4.4.4 Possible Involvement of Infection Induced Proteins in Soybean Resistance to *P. pachyrhizi*

Spots, 1-274, 1-483, 1-781, 1-1378, 1-1589, and 1-1860 were identified from comparisons with susceptible PI548631. Spot 1-274 showed high homology to lactoylglutathione lyase (commonly known as glyoxalase I), which functions in detoxification of methylglyoxal and 2-oxoaldehydes that can chemically damage several components of the cell (Chen et al. 2004). A transcript of this protein was up-regulated in sorghum during attack by phloem-feeding aphid. It also was suggested that an accumulation lactoylglutathione lyase and detoxification of ROS simultaneously occur in greenbug-stressed sorghum seedlings (Zhu-Salzman et al. 2004).

Spot 1-483 showed a high homology (83%) to 1-deoxyxylulose-5-phosphate reductoisomerase, which is involved in isoprenoids synthesis (Lange et al. 2000). Isoprenoids play essential roles in plants as hormones, photosynthetic pigments, electron carriers, membrane components, signal transduction and defense (Mahmoud and Croteau 2001). Over-expression of 1-deoxyxylulose-5-phosphate reductoisomerase in mint resulted in elevated level of isoprenoids and an increase of essential oil production (Mahmoud and Croteau 2001).

Spot 1-781 was identified as the ATP synthase subunit delta protein, which functions in producing ATP from ADP by H^+ gradient. Induction of ATPase was detected in tomato containing Cf5 resistant protein challenged by the avr5 gene products from race 4 of *Cladosporium fulvum* (Vera-Estrella et al. 1994).

Spot 1-1378 showed a high homology to the elongation factor 2, which is normally generated by pathogens as an elicitor (Dallo et al 2002; Kunze et al. 2004).

Spot 1-1589 showed high homology to a phosphoglycerate kinase (Q42961, Bringloe et al. 1996). It has been reported that nitric oxide interplays with Ca^{2+} and protein kinases including phosphoglycerate kinase to initiate nitric oxide signaling in *Arabidopsis* cell suspension culture treated with the NO-donor *S*-nitrosoglutathione (Lindermayr et al. 2005).

Spot 1-1860, a SAM-2 (S-adenosylmethionine synthetase 2), has been discussed in the previous chapter 3, in which it is involving in plant metabolisms as well as defense mechanism.

Spots, 2-728, 2-958, 2-1160, 2-1184, 2-1471, 2-1571, 2-1647, and 2-1977 were identified from comparisons with susceptible 93M60. Spot 2-728 showed a high sequence similarity (89%) to the FKBP (FK506-binding protein) type peptidyl-prolyl *cis-trans* isomerase (PPIase) 3 from *Arabidopsis* (NP_199380; Gupta et al. 2002). This protein is reported to regulate folding, assembly, and trafficking of substrate proteins, and to act as a molecular chaperone (Wang and Heitman 2005; Lima 2006). Recombinant FKBP protein cloned from Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) and over-expressed in *Escherichia coli* showed peptidyl−prolyl *cis−trans* isomerase activity and antifungal activity against pathogenic fungi, including *Candida albicans, Botrytis cinerea, Rhizoctonia solani,* and *Trichoderma viride* (Park et al. 2007). Another study showed that FKBP peptidyl−prolyl *cis−trans* isomerase was involved in protein folding, and it induced accumulation of the photosystem II (PSII) supercomplex in the chloroplast thylakoid lumen in *Arabidopsis (*Lima et al. 2006). Proteomic analysis of bean (*Phaseolus vulgaris*) infected by virulent and avirulent obligate rust fungus (*Uromyces appendiculatus*) showed that FKBP peptidyl−prolyl *cis−trans* isomerase was up-regulated in host plants infected by an avirulent race at 1 day after infection (Lee et al. 2009). This protein also was induced in resistant soybean at 1 day after *P. pachyrhizi* infection in our study. Functional diversity of FKBP peptidyl−prolyl *cis−trans* isomerase, such as protein folding, restoring PSII or antifungal activity may contribute to soybean resistance to ASR.

Spot 2-958 has a high identity (80%) to a possible membrane associated 30 kDa protein (Q03943). No studies of this protein have been published. This protein was located to the chloroplast envelope and thylakoid membranes so it may be involved in photosynthesis (Li et al. 1994). It has been reported that increased photosynthesis induced resistance of broad bean (*Vicia faba* L.) against rust (*Uromyces viciae-fabae*) (Murray and Walters 1992). When they fed $^{14}CO_2$ to the upper uninfected leaves of rusted plants, there was a high increase in labeled $^{14}CO_2$ assimilation in those leaves. In addition, substantial movement of labeled assimilate to rusted leaves was detected (Murray and Walters 1992). This result indicated that the rates of net photosynthesis were significantly increased in uninfected upper leaves following inoculation of the lower leaves.They concluded that increased photosynthesis in uninfected leaves facilitates maximum expression of resistance to rust infection.

Spot 2-1160, which is an unknown protein with 39% sequence identity to a protein phosphatase 2C (PP2C) of *Arabidopsis*, may function in plant growth, development and responses to hormones and abiotic stresses according to an earlier study by Schweighofer et al. (2007). The involvement of PP2C in regulating abscisic acid (ABA) response has been well established in *Arabidopsis* (Kerk et al. 2002), and ABA was generally considered a negative regulator of disease resistance (Mauch-Mani and Mauch, 2005). Other studies showed that increased PP2C protein resulted in disease resistance in tobacco (Hu et al. 2009) and bean (Lee et al. 2009). Rice PP2C gene over-expressed in transgenic tobacco plants lead to enhanced disease resistance and constitutive expression of defense-related genes (Hu et al. 2009). Therefore, it was suggested that OsBIPP2C2a may play an important role in disease resistance through activation of defense responses (Hu et al. 2009). Proteomic analysis of bean (*Phaseolus vulgaris*) infected by virulent and avirulent obligate rust fungus (*Uromyces appendiculatus*) showed that PP2C was up-regulated 1.6 fold in the host plant infected by an avirulent race at 1

day (Lee et al 2009) compared to the control. The up-regulation of this protein under rust infection conditions may lead to enhanced expression of defense related proteins through signal transduction. In our study, this spot was 2.3 fold and 2 fold higher in infected resistant accessions PI417089A and PI567104B compared to susceptible cultivar 93M60, respectively.

Spot 2-1184 showed a moderate sequence homology (43%) to nucleoside diphosphate kinase 2 (NDP kinase 2). NDP kinases (NDPKs) are multifunctional proteins that regulate a variety of eukaryotic cellular activities including cell proliferation, development, and differentiation, signal transduction, and phosphotrasnfrase activity (Engel et al. 1998; Galvis et al. 2001; Otero 2000). One study over-expressing *AtNDPK2* in *Arabidopsis*, resulted in high levels of autophosphorylation, NDPK activity, and low levels of reactive oxygen species (ROS) compared to wild-type plants, suggesting that this protein might be involved in enhancing plant tolerance to multiple environmental stresses (Moon et al. 2003). In rust fungus infected soybean leaves, it is speculated that increased expression of this protein might be a response to increase of ROS.

Spot 2-1471 showed a high homology (91%) to a cell division protease, ftsH homolog 2 (Yue et al. 2010). These ATP-dependent proteases have been shown to play crucial roles in repairing PSII after light-induced photodamage to prevent chronic photoinhibition (Silva et al. 2003). The up-regulation of this protein in our study might contribute to degrading damaged proteins involved in PSII system to reconstitute photosynthesis.

Spot 2-1571 showed a 65% sequence homology to the ferredoxin-NADP reductase (FNR), catalyzing the last step of photosynthetic electron transport in chloroplasts by driving electrons from reduced ferredoxin to $NADP^+$ (Hajirezaei et al. 2002). Transgenic tobacco (*Nicotiana tabacum*) plants expressing a pea (*Pisum sativum*) FNR targeted to chloroplasts exhibited an enhanced tolerance to photooxidative damage and redox-cycling herbicides

(Rodriquez et al. 2007). The upregulation of this protein in resistant soybean lines might be in response to photodamage caused by necrosis in rust fungus infected leaves.

Spot 2-1647, which completely matched (100%) to a DNA repair protein RAD23 (Ishikawa et al. 2004) with a ubiquitin-like (UbL) domain for binding to proteasome, has been shown to interact with the nucleotide excision-repair (NER) factor Rad4 and with ubiquitinated proteins to promote their degradation by the proteasome (Ortolan et al. 2004). This protein is required for conferring resistance to DNA damage (Ortolan et al. 2004). In plants, the ubiquitin/proteasome pathway of protein degradation has been implicated in plant responses to internal and external stimuli, including phytohormones, abiotic stress, and pathogen attack (Dong et al. 2006; Haglund and Dikic 2005; Smalle and Vierstra 2004). It was reported that protein polyubiquitination plays a role in basal host resistance of barley. Therefore, it is likely this RAD23 protein might be involved in soybean basal defense against rust infection.

Spot 2-1977 showed high homology to the thylakoid luminal 19 kDa protein, which is involved in photosysnthesis. Interestingly, this protein also has been reported as up-regulated in rice seedling leaves after treatment with hydrogen peroxide along with OEE1 (Wan and Liu 2008). Up-regulation of thylakoid luminal protein involved in photosynthesis along with OEE1, was crucial for PSII in this study indicating that photosynthesis might confer resistance through generating oxygen in soybean.

In addition, constitutively expressing protein spots in two resistant accessions without ASR infection (spot 91), and constitutively expressing spot only in one resistant (spot 1249.1) without infection were sequenced. Spot 1249.1 showed a high homology (100%) to the stress and rust infection induced PR 10 protein reported in Chapter 3 and spot 91 showed very high sequence homology (84%) to an oxygen evolving enhancer protein 1 (OEE1), a nuclear-encoded chloroplast protein bound to PSII on the luminal side of the thylakoid membrane that is the most

important protein for oxygen evolving in PSII (Sugihara et al. 2000; Heide et al. 2004). Increased OEE1 protein expression along with β-1, 3-glucanase and peroxidase was reported in *Vitis rotundifolia* (wild grape) tolerant to bacterial disease (*Xylella fastidiosa*) (Basha et al. 2010). It also has been reported that OEE1 gene expression was increased by abiotic stress in mangrove, *Bruguiera gymnorrhiza* (Ezawa and Tada 2009). Up-regulation of this protein in resistant accessions following inoculation in this study probably enabled host plant to generate oxygen for producing more ROS for inducing HR reaction.

Among all of the sequenced differentially expressed proteins identified in this study, two spots, 2-1471 (cell division protease ftsH homolog 2) and 91 (oxygen evolving enhancer protein 1), are involved in PSII. Especially, spot 91 was constitutively and highly expressed before inoculation in resistant accessions and it still remained high after ASR infection. Up-regulation of these proteins in resistant accessions against ASR in this study agrees with a recent study of the impact of *P. pachyrhizi* infection on soybean leaf photosynthesis and radiation absorption (Kumudini et al. 2008). They found a huge decline in carbon exchange rate (CER) as disease severity increased compared to disease-free control and concluded that soybean rust–induced reductions in CER were mainly associated with a lower efficiency of PSII photochemistry and damage to PSII reaction centers (Kumudini et al. 2008), which are commonly observed in rust fungus infected susceptible leaves.

In summary, two ASR resistant accessions, PI417089A and PI567104B, were identified by two methods, the detached-leaf assay and greenhouse inoculation. They showed an immune reaction in both assays. Detection of fungal DNA accumulation using real-time PCR showed a significantly higher fungal DNA accumulation in two susceptible accessions (PI548631 and 93M60) compared to two resistant accessions (PI417089A and PI567104B). Interestingly, a low level of fungal DNA accumulation was detected in PI567104B while no DNA accumulation was

detected in PI417089A. Differentially expressed soybean leaf protein between resistant and susceptible accessions with and without inoculation also were identified. PR10 and CHI found in a previous study were found to be up-regulated in both resistant lines and PI417089A alone, respectively. Sixteen up-regulated proteins, which only appeared in resistant accessions after infection, were sequenced. They were involved in metabolism, defense, photosynthesis, growth and development, and protein ubiquitination. Further studies involving cloning of their corresponding genes from soybean plants and characterizing their possible functions in disease resistance will be necessary to understand the exact roles that these proteins might have in soybean resistance to ASR.

CHAPTER 5
SUMMARY AND CONCLUSIONS

A 2-DE based proteomic approach was used to detect and identify differentially expressed proteins to better understand the host-parasite interaction between soybean and *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust disease. In this study, the objectives were: 1) identify host and fungal proteins induced during a compatible interaction using proteomics, 2) screen soybean accessions to identify accessions resistant to a Louisiana ASR isolate, 3) study differentially expressed proteins between resistant and susceptible accessions with and without fungal infection using proteomics, and 4) characterize these proteins to understand host-fungus interactions , and 5) verify the importance of promising host proteins in disease resistance using a virus induced gene silencing method.

Forty soybean leaf proteins differentially expressed after *P. pachyrhizi* infection during a compatible interaction were identified, and 14 of them were sequenced. Eleven of the induced proteins appear to have a role in plant defense, stress, protein modification or transport, whereas three other proteins involved plant growth and development were suppressed after rust infection. A time-course experiment showed that changes for some of the proteins were detected as early as 10 hai, whereas other proteins were induced only at a late stage of rust infection. Two such proteins, PR10 and CHI1, were further characterized because previous microarray studies also suggested that both play important roles in host response to ASR (Choi et al. 2008: van de Mortel et al. 2007). Real-time PCR results showed that gene expression pattern and protein expression pattern during the time-course were not closely related. An enzyme activity test of CHI1 showed a pattern similar to protein expression. Complementary DNAs of PR10 and CHI1 were cloned and over expressed in *E. coli*. Anti-fungal assay and RNA degradation assays have

been conducted using over-expressed PR10, but they did not show any activity in both assays. Further studies involving virus-induced gene silencing to reduce the expression of these two proteins are currently ongoing and will hopefully provide more definite answers as to their functions in soybean resistance to rust infection.

Twelve accessions were screened in this study to identify accessions resistant to a LA ASR isolate. Two resistant (PI417089A and PI567104B) accessions with immune reactions were identified through both a detached-leaf assay and greenhouse inoculation. Detection of fungal DNA accumulation using real-time PCR showed significantly higher fungal DNA accumulation in two susceptible lines (PI567204B and 93M60) compared to two resistant lines (PI417089A and PI567104B). Different levels of fungal DNA accumulation detected in the two resistant lines may indicate the presence of different defense mechanisms.

Soybean leaf proteins differentially expressed between two resistant and two susceptible accessions with and without inoculation were identified using proteomics. Eight and 15 proteins were identified as induced spots at 1 day after rust infection in both resistant accessions after comparison with PI548631 and 93M60 susceptible accessions, separately. PR10 and CHI found in the compatible interaction study also were found to be up-regulated in both resistant accessions and PI417089A alone, respectively. Sixteen up-regulated proteins which only appeared in resistant accessions after ASR inoculation were sequenced. They were involved in metabolism, defense, photosynthesis, growth and development, and protein ubiquitination. Two of the proteins, nucleoside diphosphate kinase 2 (spot 2-1184) and ferredoxin-NADP reductase (2-1571), have a function in reducing ROS stress for fortification of host cells (Moon et al. 2003; Rodriquez et al. 2007). Two other upregulated proteins, a cell division protease ftsH homolog 2 (2-1471) and an oxygen evolving enhancer protein 1 (spot 91), play an important role either in the function or in the repairing of PSII (Silva et al. 2003; Sugihara et al. 2000). This finding

agrees with an earlier report that ASR infection damaged the PSII system in the soybean plant (Kumudini et al. 2008). Further studies involving cloning their corresponding genes from soybean and characterizing their functions are necessary for better understanding of the potential roles these proteins play in soybean resistance to rust infection

Here, I have presented a detailed analysis of proteome differences between rust fungus infected and control soybean leaves, and between resistant and susceptible soybean accessions with and without inoculation based on the 2-DE coupled with MS/MS mass spectrometry. This is the first study of soybean and *P. pachyrhizi* interaction using proteomics to detect differential protein expression. In this thesis study, I found that up-regulated proteins involved in various metabolic pathways also were likely involved in the soybean defense mechanism against ASR. The failure to identify proteins involved in fungal pathogenicity was possibly due to their low abundance, or their hydrophobicity that could not be well-solubilized and separated in a standard 2-DE gel system. In a future study, we should use a more sensitive staining method to identify fungal proteins involved in soybean-rust interactions, and to characterize the up-regulated host proteins identified in this study. Knowing the molecular mechanisms that underlies the plant response to *P. pachyrhizi* will help in developing a more efficient disease control measure by enhancing host resistance using genetic engineering than the currently used fungicide applications.

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APPENDIX
VIABILITY OF *PHAKOPSORA PACHYRHIZI* UREDINIOSPORES UNDER SIMULATED SOUTHERN LOUISIANA WINTER TEMPERATURE CONDITIONS

1. Introduction

Phakopsora pachyrhizi, the primary causal agent of soybean rust disease, can infect soybean plants and cause quick defoliation and severe yield losses (7). This disease was first discovered in Japan in 1902 and later spread into China and other Asian countries. In recent years, the disease entered Africa and South America and has spread rapidly in these continents. *P. pachyrhizi* was first detected in South America in Paraguay in 2001, from where it was spread by wind across the border into Argentina. Between 2001 and 2003, it became established and widespread in soybean production regions of Brazil (19, 26). Now, soybean rust is a major emerging disease in the continental US since its discovery in late 2004 in Louisiana (20). Soybean rust disease was reported in nine, 15 and 19 states from 2005 to 2007, respectively, according to the USDA soybean rust information website (http://www.usda.gov/soybeanrust/). Based on model predictions, soybean rust disease is expected to become established in the United States, but very likely to be restricted to the southern US where the fungus could overwinter in frost-free areas or areas with brief below-freezing temperatures during the winter, such as Louisiana (9, 14, 17, 18, 25). Yield loss due to soybean rust was predicted as low as 10% in most of the United States and up to 50% in the Mississippi Delta and southeastern states in early, pre infestation models (25).

Temperature is one of the key factors affecting rust spore viability. Keogh (8) reported that urediniospores of *P. pachyrhizi* germinate at temperatures between 8 and 33°C. When temperatures were kept at 4-5°C or below, urediniospores lost their viability in 5 days (15). When temperature was raised to 9°C or higher, *P. pachyrhizi* urediniospores could remain viable

for up to 27 days (22). Kochman (10) reported significant reduced germination when dry spores were exposed to temperatures of 28.5-42.5°C. The optimum temperature for rust disease development, however, is slightly different from that for viability. Levy (11) reported that natural infection by *P. pachyrhizi* in Zimbabwe is favored by a maximum temperature of 26 to 29°C and a minimum temperature of 15 to 17°C. Under controlled environmental conditions, no infection was observed at temperatures 27.5°C or higher (12,13). Rust disease development also was greatly inhibited when mean night temperature dropped below 14°C, and was stopped when night temperature reached below 9°C (13,23). In addition to temperature, humidity also affects soybean rust disease development in the field. Melching et al. (13) indicated that humidity of over 80% for 4-6 h was necessary for disease development and urediniospores lost their infectivity completely after eight days on dry foliage.

However, the main concern for soybean growers in the US is whether *P. pachyrhizi* urediniospores can survive the winter conditions in southern US, such as Louisiana, and cause a new cycle of infection in the next growing season. Therefore, the objective of this study was to examine the viability and infectivity of soybean rust spores exposed to simulated winter conditions (12°C, 14 h day and 1°C, 10 h night with 75% relative humidity) for various durations. Additionally, over-wintered kudzu leaves were collected in January 30, 2008 from the field where soybean rust had been reported for in the past two years, to determine whether over-wintered soybean rust spores were still viable.

A.2. Materials and Methods

Materials.

Soybean rust (*Phakopsora pachyrhizi*) urediniospores were collected from infected soybean leaves in October 2006 at Central Research Station, Louisiana State University, Baton

Rouge. Spores were collected from infected soybean leaves (R8 stage) in the field using a hand-held vacuum in the early afternoon. Spores were stored at -80°C before they were used for experiments, and they were termed as frozen spores which mean 0 day or non-winter treatment in the present study. Soybean plants (cultivar 93M60, Pioneer) were grown in 20 cm diameter plastic pots (four plants per pot) in the greenhouse. The 3rd to 5th trifoliolate leaves at R1 to R2 stages were used in the detached leaf assays.

Winter treatment of urediniospores.

One mg subsamples of urediniospores from -80°C were stored in 1.5-ml microcentrifuge tubes (with lid open). The tubes were maintained either at room temperature (25°C) inside a sealed box with a relative humidity maintained at 75% using a saturated NaCl solution or under a simulated southern LA winter conditions (12 ± 1°C, 14 h day with a light intensity of 50 µE $S^{-1}m^{-2}$ and 1 ± 1°C, 10 h night with 75% relative humidity) for up to 60 days in a diurnal incubator. Winter-treated spore samples were removed daily during the first 7 days to examine the effect of short term winter treatment on spore viability. For the long term effect of winter treatment on spore viability, germ tube development, and infectivity, winter-treated spore samples were removed at 0, 4, 14, 30, 44 and 60 days from the experimental conditions and examined. The simulated southern LA winter conditions were based on the high and low average winter temperatures recorded from southern Louisiana (Cameron, Vermilion, St. Mary, and Lafourche Parishes) to central Louisiana (Vernon, Rapides, and Avoyelles Parishes) in the past 30 years during December and January (http://www.weather.com) (Table 1). The studies were conducted three times, with three replicates for each time points. Means were separated by least significant difference (LSD) test at $P = 0.05$ using the Statistical Analysis System (SAS Institute, Cary, NC; version 9.1).

Table A.1. Average high and low temperatures during December and January in southern and northern Louisiana in the past 30 years (data were compiled from http://www.weather.com).

	Southern Louisiana		Northern Louisiana	
	Av. T_{high} (mean ± SD) [y]	Av. T_{low}	Av. T_{high} (mean ± SD) [z]	Av. T_{low}
Dec.	17.1 ± 0.7	5.5 ± 1.1	14.5 ± 0.8	3.0 ± 0.8
Jan.	16.6 ± 2.6	4.4 ± 1	13.0 ± 0.9	1.8 ± 0.9

[y] Mean high and low temperature (°C) of 19 parishes in southern Louisiana between December and January: Vernon, Rapides, Avoyelles, Beauregard, Allen, Evangeline, St. Landry, Pointe, Coupee, East Baton Rouge, Tangipahoa, Washington, Calcasieu, Acadia, Iberville, Cameron, Vermilion, Iberia, and St. Charles. SD = standard deviation.
[z] Mean high and low temperature (°C) of 10 parishes in northern Louisiana between December and January: Union, Morehouse, East Carroll, Bienville, Jackson, Madison, Nachitoches, Winn, Tensas, and Grant. SD = standard deviation.

Viability of urediniospores and germ tube growth.

Soybean rust urediniospores in microcentrifuge tubes (1 mg/tube) were removed from simulated winter temperature conditions after 0, 4, 14, 30, 44 and 60 days. Spores were resuspended in 1 ml of deionized water containing 0.01% Tween 20 and allowed to germinate at room temperature for 12 h (3) along with control spores that had been kept at room temperature for the same period of time. Spore viability under different conditions was assessed using spore germination rate, which was defined as the percentage of spores germinated. At the end of incubation, the spore suspension was mixed and three 20-µl subsamples were removed from the microcentrifuge tube and examined with a microscope. The percentage of spores germinated was determined based on the total number of germinated spores versus total number of spores counted from at least 25 different fields of view (at ×200magnification) for each sample. The highest number of spores seen in a field was 44 and the lowest number was 15, with an average of 26.8 ± 6.1 spores per field. The germination percentage for each time point was the mean from

three replicated samples.

For determining germ tube growth, three 20 µl subsamples of winter-treated and frozen spore suspensions were removed from the water suspension in microcentrifuge tubes at various times ranging from 0 to 10 h and examined microscopically. Images of germinated spores from at least 25 different fields of view for each sample were captured by a Spot RT camera (Diagnostic Inc., Sterling Heights, MI) attached to the microscope. The germ tube length of each germinated spore was measured using the Spot Advance software (Diagnostic Inc.). The mean germ tube length for each of time points was determined from two repeated experiments, each with three replicated samples. Means were separated by the LSD test at $P = 0.05$.

Inoculation of detached soybean leaves with *P. pachyrhizi*.

The infectivity of rust spores which had been stored under simulated LA winter conditions for various durations (0, 4, 14, 30, 44 and 60 days) was assessed using an *in vitro* detached leaf assay. Winter-treated and frozen spores were resuspended in deionized water containing 0.01% Tween 20. Spore concentration was determined using a hemocytometer and adjusted to 2500 spores/ml. Two hundred microliters of inoculum containing 500 spores were applied evenly to the adaxial surfaces of detached soybean leaves that had been washed three times with deionized water and air-dried. Inoculated leaves were placed adaxial surface up on filter paper soaked with sterile water in Petri dishes. The inoculated leaves were incubated under the following conditions: 26 ± 0.5°C, 16 h day (about 50 µE $S^{-1}m^{-2}$) and 20 ± 0.5°C, 8 h night. Pustule formation was determined by visual inspection daily. High moisture inside Petri dishes was maintained by adding 3 ml of deionized water every 4 days. Infection rate was determined by the percentage of leaves with visible pustules versus total number of inoculated leaves. Pustule density was defined as the average number of pustules per leaf 15 days after inoculation. This experiment was conducted twice with two replicates. Each replicate consisted of 24

detached soybean leaves: half of them inoculated with rust urediniospores and the other half with water containing 0.01% Tween 20. The data from two repeated experiments were combined to calculate the mean pustule densities, and the means were separated by LSD at $P = 0.05$.

Viability of soybean rust spores recovered from over-wintered kudzu leaves.

Over wintered dry kudzu leaves were collected in Jan. 30, 2008 from the two locations in southern Louisiana (New Iberia, LA) where soybean rust on kudzu had been reported in the past two consecutive years. These earlier infected kudzu leaves had senescenced at the end of growing season and fallen off vines during the winter. The collected leaves were first examined with a dissecting microscope (Leica MZ16) to confirm pustule lesions at ×200 Leaves with lesions were then sliced into 3-by-5 mm sections and transferred to a 15-ml centrifuge tube with deionized water containing 0.01% Tween 20 enough to submerge all leaf sections. After 12 h incubation at room temperature, the spore suspension was examined with a microscope for viability.

3. Results

Effect of simulated southern Louisiana winter temperature conditions on *P. pachyrhizi* urediniospore viability.

The average germination rate of urediniospores freshly harvested from the field varied greatly from 93% to 15% depending on the time of harvest and the micro-environment which the spores were exposed to before harvest (Park and Chen, unpublished data). Spores can be stored at -80°C for up to one year without showing a further decline in germination rate (5,21). The spores used for this study had an average germination rate of 72% to 80%. Frozen soybean rust urediniospores stored at room temperature (25°C) lost their viability gradually from 72% to 32% in 7 days, whereas the viability of spores stored under simulated winter conditions decreased from 72% to 40% in the first 24 h, followed by a steady decrease to about 17% at the end of 7

days (Fig. A.1). Spores kept at room temperature had a significantly higher viability than that of winter treated spores after 2 days of storage (Fig. A.1). However, the difference in viability between spores kept at room temperature and under winter conditions diminished as the time increased and became insignificant at day 7 (Fig. A.1).

In an effort to determine how long soybean rust spores remain viable under simulated southern Louisiana winter temperature conditions, the germination rate of spores was examined bi-weekly up to 60 days in a separate experiment. It was found that spore germination rate decreased rapidly from 72% to about 22% in 14 days, and then more slowly to 11% at 60 days when stored under simulated winter temperature conditions (Fig. A.2). However, the germination rate of control spores kept at room temperature decreased from 76% to 32% in the first two weeks (Fig. 2), and then decreased steadily to 20% and 8% at 30 days and 44 days, respectively. Spore germination rate reached 0% at the end of this 60-day study (Fig. A.2).

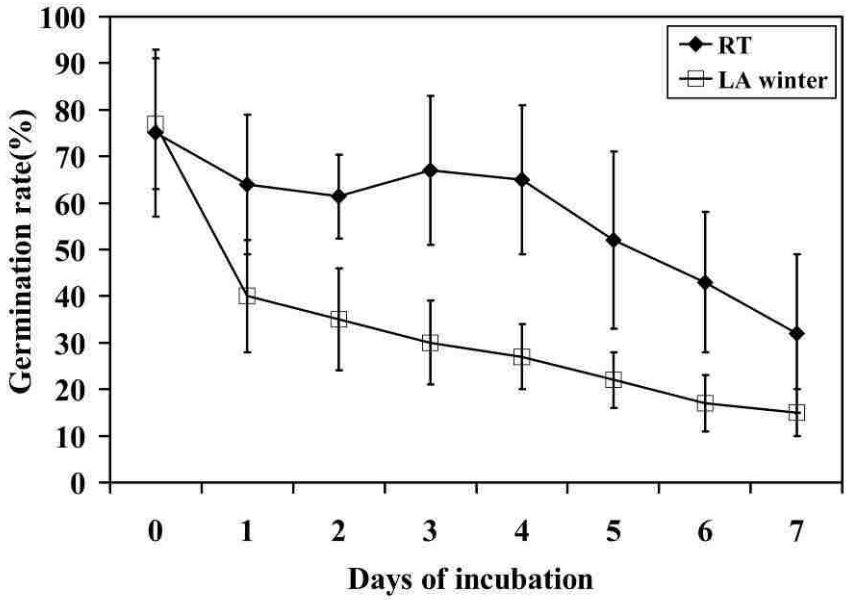

Figure A.1. The effect of short-term winter temperature treatment on the germination rate of *Phakopsora pachyrhizi* urediniospores. Germination rate of winter-treated spores (up to 7 days) was compared daily to control spores kept at room temperature for the same duration. Germination rate was measured as the percentage of spores germinated at room temperature after being suspended in deionized water containing 0.01% Tween 20 for 12 h. RT, room temperature (25-26°C); LA winter, simulated southern Louisiana winter conditions (12°C, 14 h day and 1°C, 10 h night with 75% relative humidity). Vertical bars represent standard deviation.

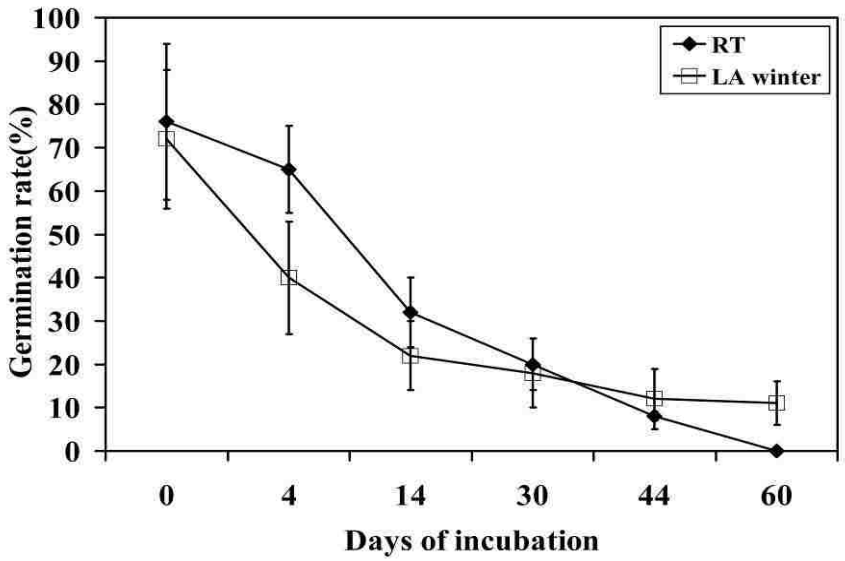

Figure A.2. The effect of long-term winter temperature treatment on the germination of *Phakopsora pachyrhizi* urediniospores. Germination rate of winter-treated spores (up to 60 days) was compared to control spores kept at room temperature for the same duration. Germination rate was measured as the percentage of spores germinated at room temperature after being suspended in deionized water containing 0.01% Tween 20 for 12 hr. RT, room temperature (25-26°C); LA winter, simulated southern Louisiana winter conditions (12°C, 14 h day and 1°C, 10 h night with 75% relative humidity). Vertical bars represent standard deviation.

Effect of simulated winter temperature treatment on spore germ tube growth.

Simulated-winter temperature treatment not only reduced spore viability, but also slowed spore germ tube growth (Fig. A.3). Germ tube development for frozen spores was clearly visible after 2 h of germination and elongated rapidly between 4 to 8 h. Germ tube length reached an average of 90 µm after 4 h and an average of 250 µm at the end of 10 h of incubation (Fig. A.4). Germ tube length of winter-treated spores was significantly shorter than frozen spores after 2 h of germination except for the spores that were winter-treated for only 4 days (Fig. A.4) The average germ tube length was 30 µm at the end of 10 h germination for the spores that had been treated for 14 days or longer, which was about 8 times shorter than those of frozen spores (Fig. A.4). The average germ tube growth rate for frozen spores and spores under winter conditions for 4 days was about 25 µm/h compared to that of 3 µm/h for the spores that had been under winter temperature conditions for 14 to 60 days.

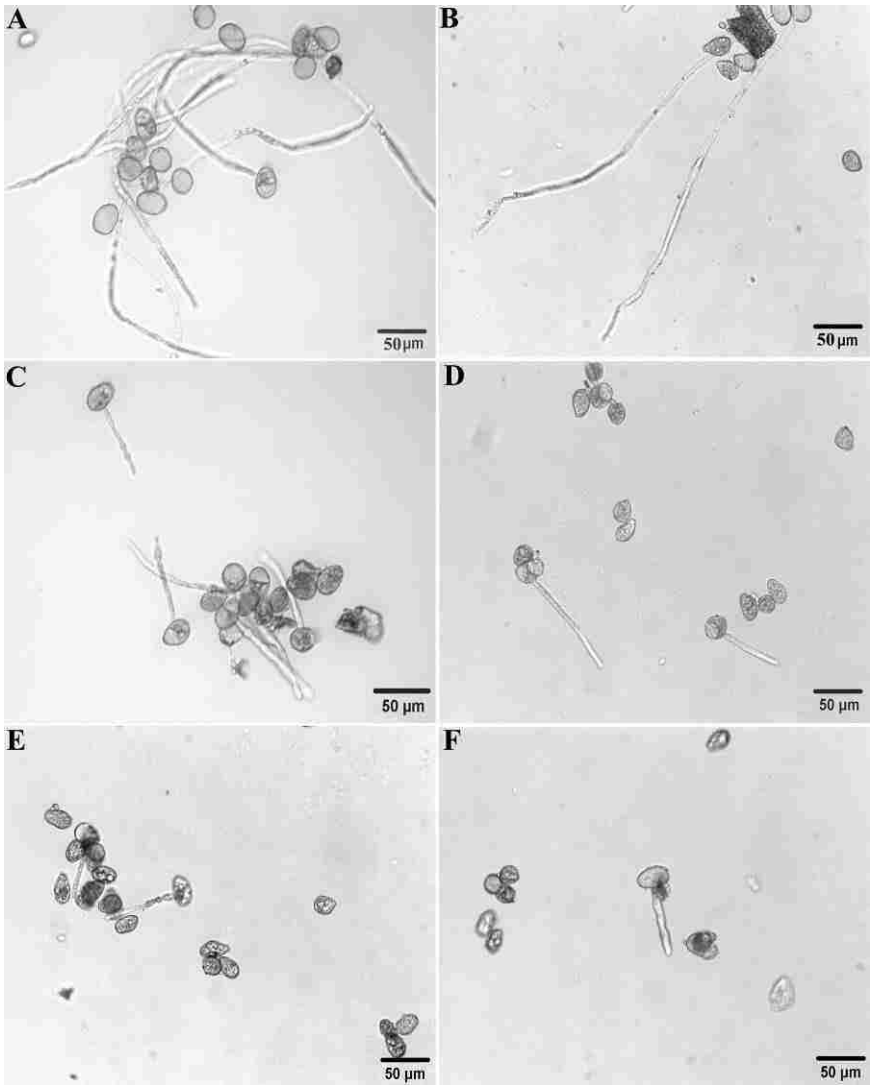

Figure A.3. The effect of simulated winter temperature treatment on *Phakopsora pachyrhizi* urediniospore germ tube development. Germ tube growth was examined after incubating frozen spores and over-wintered rust spores in deionized water containing 0.01% Tween 20 at room temperature for 10 h. (A) frozen spores; (B) to (F), spores that had been under simulated winter conditions for 4, 14, 30, 44 and 60 days, respectively.

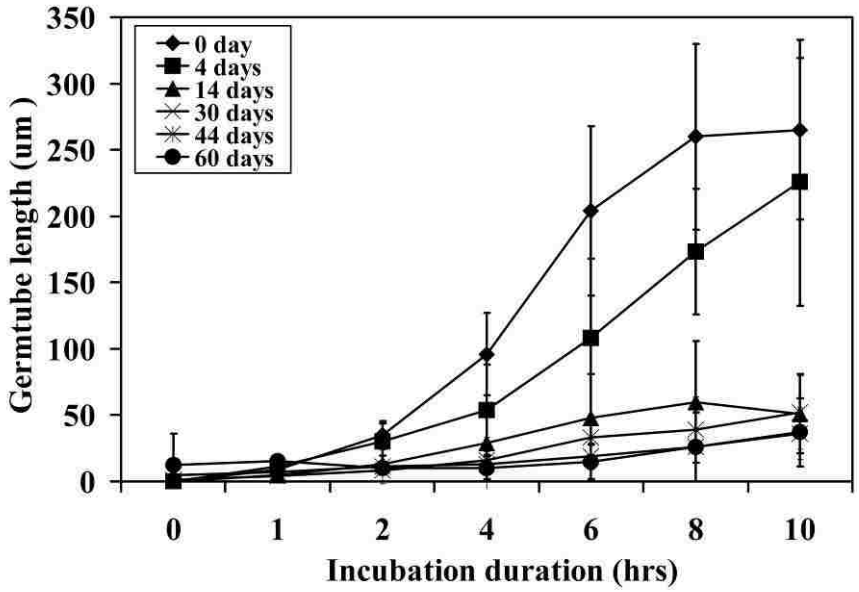

Figure A.4. The effect of winter treatment on germ tube growth rate of *Phakopsora pachyrhizi* urediniospores. Germ tube length was measured witha light microscope hourly after suspending the frozen and winter-treated spores in deionized water containing 0.01% Tween 20 at room temperature. The data presented here were means from two repeated experiments, each with three replicates. Vertical bars represent standard deviation.

Effect of winter-treatment on spore infectivity using a detached leaf assay.

Detached soybean leaves started producing roots about 7 days after incubation under the detached leaf assay conditions, enabling leaves to remain green up to 30 days after inoculation (Fig. A.5). Spores that had been treated under simulated winter temperature conditions for as long as 60 days retained their infectivity and were able to produce new pustules when inoculated onto detached soybean leaves (Fig. A.6) although the number was significantly less compared to that produced by frozen spores (Table A.2). Pustules were observed 9 days after inoculation on all soybean leaves inoculated with frozen or 4-day-old over-wintered spores (Table A.2). For leaves inoculated with 14 or 30-day-old over-wintered spores, the initial pustules were observed 9 days after inoculation, but only in 85 or 25% of the inoculated leaves, respectively. Infectivity decreased as the duration of winter-treatment increased. Leaves inoculated with spores that had

over-wintered for 44 and 60 days did not develop pustules until 12 days after inoculation, and pustules were observed in only 40% and 10% of the inoculated leaves, respectively (Table A.2).

In addition, pustule density in inoculated leaves decreased as the duration of winter-treatment increased. Fourteen days after inoculation, leaves inoculated with frozen or 4-day-old over-wintered spores had an average of 42.3 or 49.2 pustules per leaf, respectively (Table A.2). However, the pustule density was significantly lower in leaves inoculated with spores over-wintered for 14 days or longer compared to leaves inoculated with frozen spores. On the average, only 16 and 3 pustules per inoculated leaf were observed in leaves inoculated with spores that over-wintered for 14 and 30 days, respectively. Leaves inoculated with spores over-wintered for 44 or 60 days had an average of less than one pustule per inoculated leaf (Table A.2).

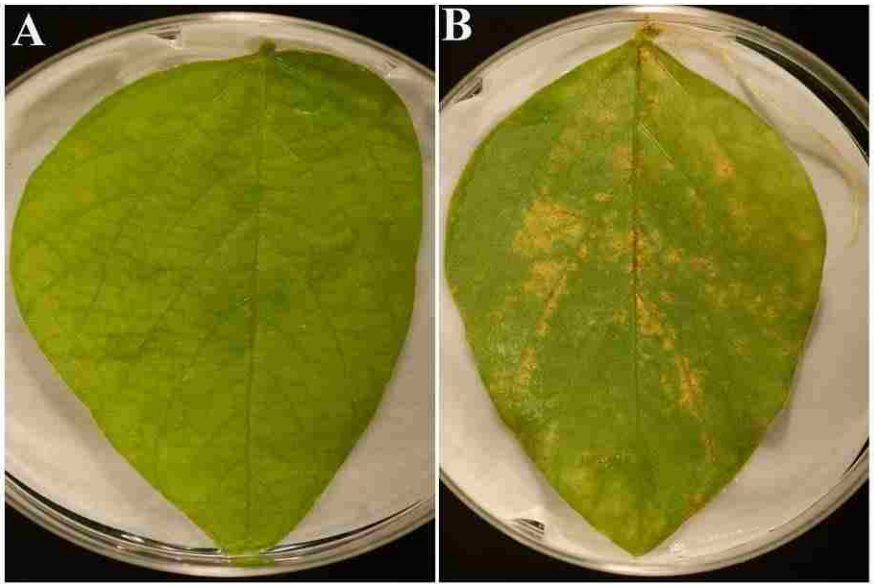

Figure A.5. Evaluation of *Phakopsora pachyrhizi* urediniospore infectivity using a detached leaf assay. Soybean leaves (3^{rd} to 5^{th} trifoliate) at R1 to R2 stage were harvested from greenhouse-grown 93M60 soybean plants, inoculated with soybean rust spores, placed on filter paper soaked with deionized water, and incubated for 14 days under the condition of 26°C, 16 h day and 20°C, 8 h night before being evaluated for disease severity or pustule density. (A), leaf before inoculation; (B) leaf 14 days after inoculation. Root formation was evident in the detached leaves after one week of incubation.

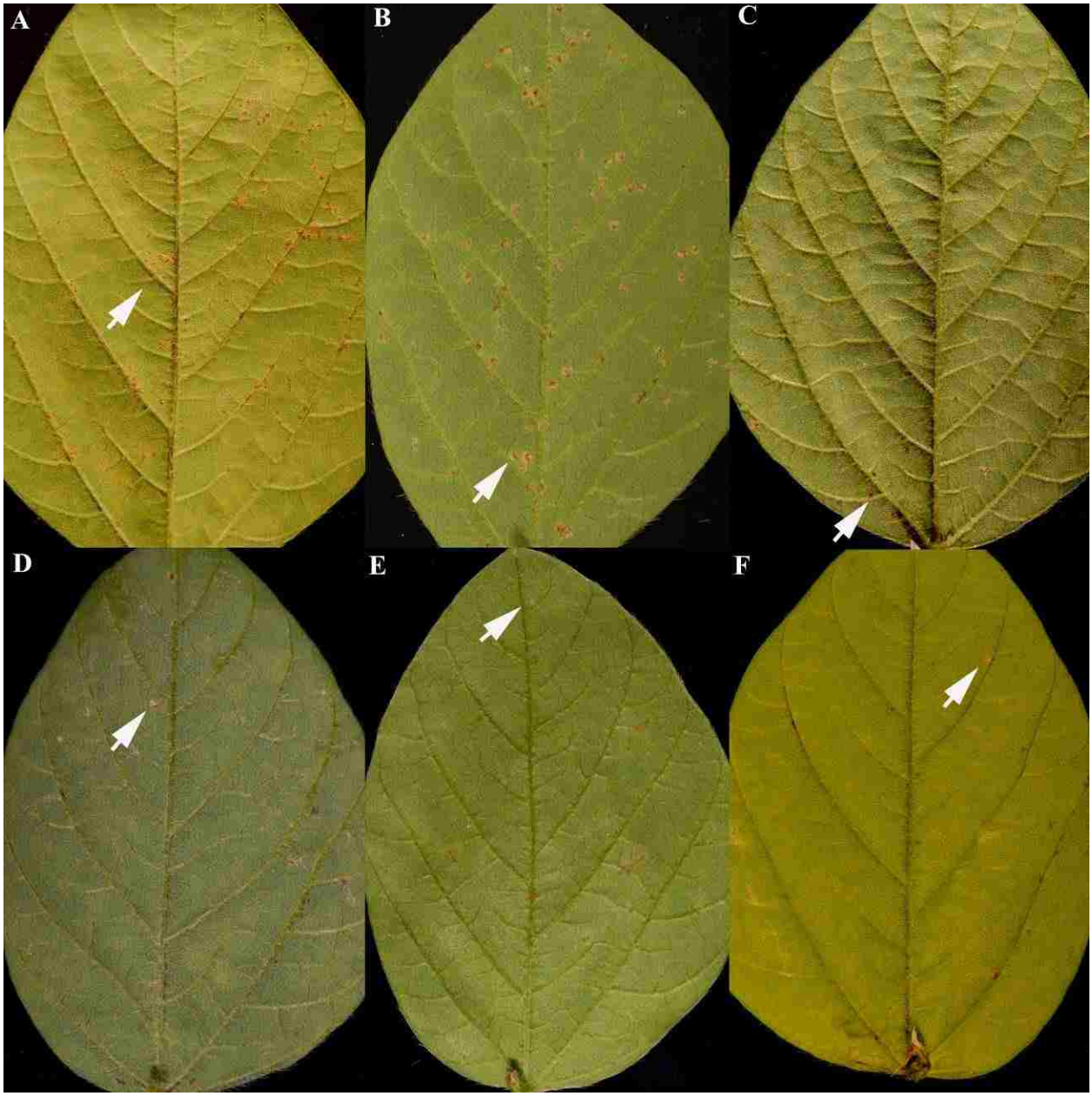

Figure A.6. The effect of winter temperature treatment on infectivity of soybean rust urediniospores. Detached soybean leaves were inoculated with rust spores that had been treated under simulated southern Louisiana winter condition for different durations. Soybean leaves were inoculated with spores over-wintered for 0 day (frozen spores, A), 4 days (B), 14 days (C), 30 days (D), 44 days (E) and 60 days (F), respectively. Photos were taken 14 days after inoculation. Arrows indicate pustules.

Table A.2. Effect of simulated winter temperature treatment on *Phakopsora pachyrhizi* urediniospore infectivity.

Duration at winter conditions	Infection rate (%)[y]			Pustule density [z] (mean ± SD)
	9 DAI	12 DAI	14 DAI	
0 day	100	100	100	42.3 ± 28.7 a
4 days	100	100	100	49.2 ± 31.8 a
14 days	85	100	100	16.4 ± 9.3 bc
30 days	25	70	85	3.3 ± 4.6 c
44 days	0	40	50	1.5 ± 2.4 c
60 days	0	10	30	0.4 ± 0.8 c

[y] Infection rate was the average percentage of inoculated leaves developing visible pustules at the specified time intervals. DAI, days after inoculation.

[z] Pustule density was the mean number of pustules per leaf observed 15 days after inoculation from two combined experiments. Means in the same column followed by a common letter were not significantly different by LSD test at $P = 0.05$; SD = standard deviation.

Viability of soybean rust spores on over-wintered kudzu leaves.

No viable soybean rust spores were recovered from kudzu leaves collected from one location. However, sixty-seven out of about 500 spores recovered from an over-wintered dry kudzu leaf at the other location were found to germinate after 12 h of incubation in water containing 0.01% Tween 20 (Fig. A.7). In addition, germ tube growth of these viable spores reached an average of 25 ± 4.7 after 10 h of germination, which was about same as those of spores that had been treated under simulated winter for 60 days.

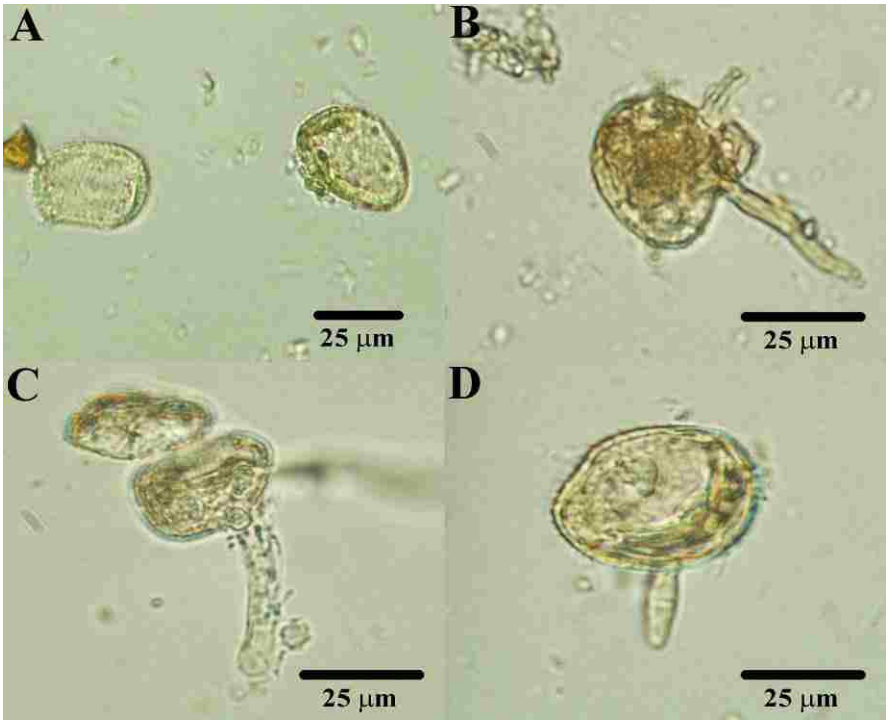

Figure A.7. Germination of *Phakopsora pachyrhizi* spores recovered from over-wintered dry kudzu leaves collected from southern Louisiana. Spore germination was examined a light microscope. A, spores before incubation; B-D, spores after 12 h incubation.

4. Discussion

Soybean rust urediniospore survivability under winter conditions, especially in the south, is a major concern for the US soybean growers. Previous studies determined the maximum and minimum temperature and moisture conditions for spore germination and infection (10,12,13). It was also reported that when temperatures were kept at 4 to 5°C lower,urediniospores lost their viability in 5 days (15). However, it has not been investigated how well soybean rust spores over-winter in the southern United States where winter night temperature is usually above 0°C. As a first step, a simulated winter condition based on average day and night temperatures from central to southern Louisiana during the past 30 years was used to treat frozen spores for various durations before examining their viability and infectivity.

In agreement with earlier studies (12,15), soybean rust spore viability was found to be detrimentally affected by low temperature treatment. The effect of simulated winter temperature

conditions on spore viability was observed within the first 24 h and viability was significantly decreased after 2 days compared spores kept under room-temperature conditions. This sudden initial decline in spore viability might be related to age variations among the collected spores since it had been previously reported that viability of spores from inactive pustules was significantly lower than that of spores from active pustules when exposed to freezing temperatures (16). It also appeared that spores kept under simulated winter conditions remained viable longer than spores stored at room temperature. It has been reported that cellular metabolism is reduced at sub-optimal temperatures (6). This may explain why spores with limited nutrient and energy reserves survived longer under simulated winter conditions than under room temperature.

Our study also found that simulated winter treatment slowed germ tube growth. The average germ tube growth for spores that had been under winter conditions for 14-60 days was about 8-fold slower than that of frozen spores. This may be why those spores were less effective than frozen spores in producing pustules when inoculated onto detached soybean leaves. The time of initial symptom appearance, infection rate, and pustule density were delayed or reduced in leaves inoculated with spores that had been treated under simulated winter conditions for 44 days or longer. However, it was demonstrated that even spores overwintered for 60 days were able to infect soybean leaves and produce pustules. It indicated that *P. pachyrhizi* urediniospores could over-winter in southern Louisiana and initiate a new cycle of infection in the next growing season, although the initial infection cycle may take longer than 14 days.

The spores over-wintered for 4 days had a similar germ tube growth rate as, but a significantly lower germination percentage (35%) than, frozen spores (72%). In the detached leaf assay, both kinds of spores showed the same infectivity. This suggests that germ tube growth rate is a more important factor than spore viability in determining whether a successful infection can

occur. This may be due to the fact that *P. pachyrhizi* spores have only 6-8 h to germinate, elongate a germ tube and penetrate the host cell wall (13) before spores exhaust their limited nutrients and energy reserves (4). A study by Adendorff and Rijkenberg (1) reported that germ tubes of direct penetrating fungi, like soybean rust, prefer the junction area between two leaf epidermal cells and penetration usually occurs 6 h after inoculation. Therefore, it is likely that spores with fast elongating germ tubes will have a better chance in finding an appropriate surface area for penetration than spores with slow growing germ tubes before the window of opportunity elapses.

A new detached leaf assay was used in the present study to examine changes in spore infectivity after the winter treatment. The earlier detached leaf assay developed by Burdon and Marshall (2) and modified by Twizeyimana et al. (24) uses 1% agar plates supplemented with 10 mg/l kinetin to delay leaf senescence. Also, the earlier assay uses only a small section of a leaf per Petri dish. Our method, first reported by Chen et al. (3), uses whole leaves, placed on sterile filter papers pre-soaked with 4 ml of sterile water per 100 mm Petri dish without agar medium or kinetin. Another difference is that the detached leaves in this new assay were incubated under light and temperature settings of 14 h day (at 26°C) and 10 h night (at 20°C). Detached leaves remained green for over a month. In addition, detached leaves in this new assay often develop roots during the first 10 days of incubation, which further delays leaf senescence. This assay proved very useful not only in determining spore infectivity in a short time, but also in maintaining live soybean rust cultures under laboratory conditions. This assay could also be used to evaluate host resistance levels of different soybean varieties under laboratory conditions.

In summary, soybean rust spores that had been stored under simulated LA winter temperature conditions for as long as 60 days germinated, infected detached soybean leaves and produced pustules though at a lower rate and density compared to frozen spores. This study

suggests that *P. pachyrhizi* could survive winter temperatures in southern Louisiana and other southern states, and serve as a source of inoculum for the coming season in North America. This conclusion is supported by the observation of viable soybean rust spores recovered from over-wintered dry kudzu leaves collected in January 30, 2008 from southern Louisiana, where night temperatures dipped four times below-freezing (-1ºC) in January alone. It is also supported by the fact that the first two 2007 soybean rust infections in Louisiana were reported on newly grown kudzu leaves at the two locations where soybean rust was reported in 2006 even though all of the earlier infected kudzu leaves and vines had died back during the 2005-2006 winter (www.sbrusa.net).

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VITA

Sunjung Park was born in Chon-Ju, Korea, in March, 1973. Her hometown is renowned for delicious food and famous scholars. She graduated from Seoul Women's University, the Department of Horticultural Science, with a Bachelor of Science degree in agriculture in 1997. She enrolled in the Department of Horticultural Science, Graduate School of Natural Resources, Korea University, in March 1997. She worked as a teaching assistant for a year while working toward the Master of Science degree. She graduated with the master's degree in horticultural science in 1999. The master's thesis is entitled "The Effect of Chitosan on Regeneration and Transformation of Chrysanthemum (*Dendranthema grandiflora*)".

After the master's degree, she worked as a research scientist at Natural Resources Institute, Korea University, from December 1998 to August 1999, at Life and Environment Science Laboratory of Kumho Petrochemical Co., Ltd., from February to July 2000, and in the Laboratory of Agricultural Postharvest Preservation of the Biosystech Research Institute, Biosystech Co., Ltd., from July 2000 to July 2001. The topics of her research were cDNA sequence from leaves of hibiscus (*Hibiscus syriacus*, National Flower of Korea), overexpression of the *Pra*2 gene in *E. coli*, and postharvest physiology to prolong the vase life of cut flowers by the MA (modified atmosphere) package method, respectively.

In 2002, she joined to the Department of Plant Pathology and Crop Physiology at Lousiana State University to pursue her master and doctoral degrees. While she worked as a graduate research assistant in the laboratory of Dr. Norimoto Murai, she worked on phaseolin promoter analysis and tobacco leaf tissue transformation under various conditions. After she finished her master's thesis titled "*Agrobacterium tumefaciens*-mediated Tansformation of Tobacco (*Nicotiana tabacum* L.) Leaf disks: Evaluation of the Co-cultivation Conditions to Increase *β*-

glucuronidase gene activity", she moved to laboratory of Dr. Zhi-Yuan Chen to study host-parasite interaction between soybean and *Phakopsora pachyrhizi* using proteomics. While she pursued her doctoral degree under Dr. Zhi-Yuan Chen, she has had opportunity to learn proteomics as well as various molecular biological techniques and biochemistry analysis. She participated in various conferences to present her findings. She also worked as teaching assistant for a general plant pathology course and as chair of social of the Plant Pathology and Crop Physiology Graduate Student Association. She will receive the degree of Doctor of Philosophy at the spring 2010 commencement.