2013

Proteomics-based study of host-fungus interaction between soybean and Phakopsora pachyrhizi using recombinant inbred line (RIL) derived sister lines

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PROTEOMICS-BASED STUDY OF HOST-FUNGUS INTERACTION BETWEEN SOYBEAN AND *PHAKOPSORA PACHYRHIZI* USING RECOMBINANT INBRED LINE (RIL) DERIVED SISTER LINES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Plant Pathology and Crop Physiology

by
Mala Ganiger
B. Sc., University of Agricultural Sciences, 2004
M. Sc., University of Agricultural Sciences, 2007
December 2013

This work is dedicated to my

Dear Mother, SMT. PRAMILA GANIGER

Dear Father, SHRI. CHANDRASHEKHAR GANIGER

Late Brother, MANNABASAVRAJ GANIGER

Loving Husband, Dr. ASHOK KUMAR CHANDA

Little Angel, HAMSINI CHANDA

ACKNOWLEDGEMENTS

I would like to convey my sincere thanks to my major advisor Dr. Zhi-Yuan Chen for his support and guidance throughout the graduate program. I would also like to thank my committee members, Drs. Christopher Clark and Rodrigo Valverde for their support and spending their valuable time with me for discussing research problems. I also would like to thank Drs. Raymond Schneider, Kenneth Damann, David Walker for partly serving on my committee and their honest criticism.

My sincere thanks to Drs. Lawrence Datnoff, Clayton Hollier, Edward McGawley, and Raghuwinder Singh for their encouragement and mental support. I really need to thank my two American mamas Patricia Bollich and Dolores Dyess who have always given me and my family so much love and support.

I would like to thank all my PPCP friends who have always given me a timely help and support. My respectful thanks to my loving parents who are dedicated to me. Finally, my sincere and heartfelt thanks to my husband Dr. Ashok Kumar Chanda who has been a great mentor to me and for his incredible support throughout my life. Thank you so much for walking every step of life with me and making me to keep my head held high in difficult times. Thanks to my little angel Hamsini, whose face always brings me so much joy and makes me to forget all worries and tiredness.

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LIST OF ABBREVIATIONS

aa	Amino acid
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ASR	Asian soybean rust
CBB	Coomassie brilliant blue
CHAPS	3-[(3- cholamidopropyl) dimethylammonio]-1-propane sulfonate
CHI	Chalcone isomerase
CHR	Chalcone reductase
DAP	Days after planting
DD	Dihydrolypoyl dehydrogenase
2-DE	2-Dimensional electrophoresis
2-DGE	2-Dimensional gel electrophoresis
2D-DIGE	2-Dimensional difference gel electrophoresis
DIGE	Difference gel electrophoresis
dpi	Days post inoculation
DTT	Dithiotheritol
ESI IT MS/MS	Electrospray ionization ion trap tandem mass spectrometry
GDH	Glutamate dehydrogenase
GLO	Glyoxylate oxydase
hai	Hours after inoculation
HR	Hypersensitive response
HSD	Honestly significant difference test

Hsp	Heat shock protein
IAA	Iodoacetamide
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
ITS	Internal transcribed spacer
kDa	Kilodalton
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MDH	Malate dehydrogenase
2-ME	2-Mercaptoethanol
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MS	Mass spectrometry
MW	Molecular weight
NCBI	National Center for Biotechnology Information
NILs	Near isogenic lines
NL	Nonlinear
NO	Nitric oxide
OEE	Oxygen evolving enhancer
p*I*	Isoelectric point
pkl	peak list file
PMT	Photomultiplier tube
PTGS	Post-transcriptional gene silencing
PTM	Post-translational modification
PMF	Peptide mass fingerprinting
PSII	Photosystem II

Q-TOF	Quadrupole time-of-flight
RuBisCO	Ribulose bisphospahte Carboxylase Oxygenase
RH	Relative humidity
RI	Rust index score
RIL	Recombinant inbred line
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHMT	Serine hydroxymethyltransferase
SSR	Simple sequence repeats
TCA	Tricarboxylic acid cycle
TMG	Tropical Melhoramento & Genética company

ABSTRACT

Phakopsora pachyrhizi, the causal agent of Asian soybean rust (ASR), has the potential to cause severe yield losses as all United States commercial soybean varieties are susceptible. In this study, 10 soybean recombinant inbred line (RIL) derived sibling lines of two populations (RN06-32-2 and RN06-16-1) were evaluated for differences in response to infection by *P. pachyrhizi*. These lines, which had previously shown differential responses to Florida soybean rust isolates, were evaluated using Louisiana soybean rust isolates under both detached leaf assay and greenhouse *in planta* inoculation conditions. Sibling lines showed significant differences in response to *P. pachyrhizi* infection under both conditions. Lines 8-a, 8-b, 94-c of population RN06-32-2 and lines 15-b and 16-c of population RN06-16-1 showed a resistant response against Louisiana rust isolates in comparison with the immune response against Florida rust isolates. Whereas, lines 15-c and 16-b of population RN06-16-1 and lines 8-c, 94-a, and 94-b showed similar responses against Louisiana rust isolates as that of Florida rust isolates. Lines 15-c and 16-b showed moderately resistant response; lines 8-c, 94-a, and 94-b showed susceptible and resistant response, respectively. To understand the compatible and incompatible host-pathogen interactions at the molecular level, we conducted a time-course study (0 h, 10 h, 1 d, 2 d, 3 d, 4 d, 5 d, 8 d, 10 d, 12 d and 14 d) of *P. pachyrhizi* infection and compared protein profiles of 8-a (resistant) and 8-c (susceptible) lines in response to ASR inoculation, using DIGE proteomics. Based on the gel analysis, we observed approximately 100 differentially expressed spots between 8-a and 8-c lines. Among these, 37 proteins were identified using mass spectrometry. Most of the identified

proteins are involved in photosynthesis and carbon metabolism, defense mechanism, seed storage and include some uncharacterized proteins.

CHAPTER 1: REVIEW OF LITERATURE

Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* was first described in Japan in 1902, and has since spread throughout the world (Ono et al., 1992). Until recently the pathogen was distributed in East Asia and Australia (Dorrance et al., 2007; Pivonia and Yang, 2004; Pivonia and Yang, 2006). The first report of *P. pachyrhizi* in the United States was on a farm in Hawaii in 1994 (Killgore et al., 1994). In the late 1990's, ASR was reported in Africa and it was reported in South America in 2001 (Yorinori et al., 2005). As of 2004, ASR has been reported in Bolivia, Brazil, Paraguay and Uruguay. In November 2004, for the first time *P. pachyrhizi* was reported in Louisiana and other southeastern states (Schneider et al., 2005).

The threat of widespread infections of soybean (*Glycine max*) fields during the growing season (July to November in the United States) has increased in the past few years, since, *P. pachyrhizi* has a wide host range and is capable of overwintering on a number of alternative hosts, including kudzu (*Pueraria lobata*), leading to high inoculum accumulation. The disease is mostly restricted to the Southern United States, primarily because of the favorable environmental conditions such as large frost-free areas or short below-freezing temperatures in the winter such as Louisiana that are favorable for ASR establishment (Kim et al., 2005; Pan et al., 2006; Park et al., 2008; Pivonia and Yang, 2004; Pivonia and Yang, 2005; Yang et al., 1991). In addition, other environmental conditions, such as temperatures ranging between 15-26 °C and humidity as high as 80% also promote ASR establishment (Levy, 2005).

Currently, the disease is mainly controlled through fungicide applications as there are few resistant cultivars available, such as the INOX cultivars from Brazil. Cultural practices like wide row spacing, adjusting soil fertility, are also effective in minimizing the ASR-related losses (Rupe and Sconyers, 2008). The efficacy of many fungicides in controlling soybean rust was evaluated in Taiwan and Japan (Hung and Liu, 1961; Kitani et al., 1960). It was shown that spraying fungicides like Plantvax®, Benlate® and Tecto® at recommended rates had no effect on improving yield but decreased defoliation (Sangawongse, 1973). The effectiveness of Mancozeb® was reported in 1992 (Hartman et al., 1992), however, the yield protection was inconsistent and varied by different application rates. Several triazole compounds and triazole mixes were also evaluated in India (Patil and Anahosur, 1998) and other countries around the world for their efficacy against rust (Miles et al., 2003b). Fungicide applications during early reproductive stages have shown protection throughout crop maturity. Recently, the concentration, number and the time of application of fungicides have also shown to be critical in controlling ASR (Miles et al., 2007; Mueller et al., 2009). Fungicides applied during the vegetative growth stages [28 days after planting (DAP)] did not increase yield compared to applications from flowering through beginning of seed filling (48 and 68 DAP). Based on this, three sprays (50, 70, and 90 DAP), and four sprays provided total rust control (Miles et al., 2003a). However, fungicide applications did not provide any economic or yield advantages. It only helps in stabilizing yields in the presence of disease, by offering protection. Also, these new fungicides often require new and expensive sprayers, and therefore, this approach of rust control is not considered as cost-effective or viable.

In addition, continuous increase in the use of fungicides has led to several serious problems, such as fungicide resistance and toxicity to non-target organisms. Resistance to newer compounds including benzimidazoles, dicarboximides, phenylamides and strobilurins has been reported in some fungal strains but not in *P. pachyrhizi* in FRAC, 2010 (Fungicide Resistance Action Committee). Azoxystrobin, a strobilurin compound used for controlling rust, also has high toxicity to aquatic organisms although it has low toxicity to other non-target organisms (Fernandez-Ortuño et al., 2008). Therefore, the run-off fungicides can have a significant negative impact on aquatic creatures in streams or ponds near the fields sprayed with the fungicides (Ochoa-Acuña et al., 2009). An independent study further found the same fungicide caused significant toxicity to mammalian cells based on laboratory assays (Daniel et al., 2007). For these above reasons, improving host resistance of soybeans to ASR is considered the most viable alternative approach to fungicide applications.

In an effort to identify soybean lines with resistance to rust, six *Rpp* genes conferring single gene resistance to ASR have been reported (Bromfield and Hartwig, 1980; Hartwig, 1986; Hymowitz, 1980; McLean and Byth, 1980a): *Rpp1* identified in soybean genotype PI200492 (Cheng and Chan, 1968; Hartwig and Bromfield, 1983; Hidayat and Somaatmadja, 1977), *Rpp2* in PI230970 (Hidayat and Somaatmadja, 1977), *Rpp3* in PI462312 (Singh and Thapliyal, 1977); *Rpp4* in PI459025 (Hartwig, 1986), *Rpp5* in PIs200487, 200526 and 471904 (Garcia et al., 2008) and *Rpp6* in PI567104B (Li et al., 2012). These genes conferred resistance only against specific isolates of *P. pachyrhizi* collected internationally or in the USA (Bonde et al., 2006; Paul and Hartman, 2009; Pham et al., 2009). Difference between the resistant and susceptible response was found

to be during early hyphal penetration, spread of hyphae and haustorial development (McLean and Byth, 1981). However, single gene resistance has not been durable and partial resistance is difficult to work with. It was shown that the effectiveness of these resistances can be overcome by virulent ASR isolates collected from other places (Hartman et al., 2005). In addition, none of these single resistance genes *Rpp1*, *Rpp2*, *Rpp3, Rpp4, Rpp5* or *Rpp6* appeared to provide strong and consistent resistance to soybean rust (Pham et al., 2010) though the resistance expressed by *Rpp2* appeared to be the most consistent (Pham et al., 2010). Therefore, developing durable genetic resistance against ASR has been difficult. Part of the reason is that there are high genetic variations among different populations of *P. pachyrhizi*. Freire et al. (2008) sequenced ITS1 and ITS2 regions of *P. pachyrhizi* isolates from 26 soybean fields and identified 27 and 19 ribotypes, respectively. Brazilian isolates shared similarity with Asian and African *P. pachyrhizi* isolates, indicating common ancestry and confirming the speculated long-distance dispersal of isolates. They also found some isolates that are unique to Brazil. In another study, 84 distinct genotypes were identified from three zones based on simple sequence repeat (SSR) analysis of 115 *P. pachyrhizi* isolates from four agroecological zones in Nigeria (Twizeyimana et al., 2011). The majority of the genetic diversity was observed within each soybean field compared to among soybean fields within geographical region. Similar results in a recent study (Akamatsu et al., 2013) showed that *P. pachyrhizi* populations from South America vary geographically and temporally.

In order to identify other sources of resistance, many of the wild perennial species of *Glycine* have also been screened for resistance against ASR (Burdon and Marshall, 1981; Hartman et al., 1992). After identification, inheritance of resistance was examined by

making intra-specific crosses. For example, *G. canescens* was found to have single dominant resistance genes in more than four loci (Burdon, 1988), and *G. argyrea* was found to have one dominant resistance gene (Jarosz and Burdon, 1990). The number of dominant resistance genes to soybean rust also varied with the number of chromosomes (2n=38, 40, 78 and 80) of different populations of *G. tomentella* (Hymowitz, 1995; Schoen et al., 1992). However, crosses made between these lines and cultivated species have not been successful, except for some crosses with *G. tomentella* (Hymowitz, 1995; Patzoldt et al., 2007; Zou et al., 2004). Since the resistance conferred by many of these perennial species has yet to be studied, there is more room for exploring the use of these genes in developing rust-resistant soybeans (Chung and Singh, 2008; Hymowitz, 1995; Soria-Guerra et al., 2010) .

Recently, molecular based approaches have been used to understand the mechanisms of host-pathogen interactions and to identify the genes involved in host defense response to ASR. The first microarray analysis of host response to ASR done by Panthee et al. (2007) showed up-regulation of general defense-related and stress-related genes indicating involvement of a low and nonspecific innate immune response. An extensive microarray analysis was conducted to study the resistance response governed by the *Rpp2* gene (van de Mortel et al., 2007). Gene expression was found to be biphasic in both resistant and susceptible plants in response to *P. pachrhizi* infection with most genes up-regulated at 12 hours after inoculation (hai). The expression profile of differentially expressed genes in the first 12 hai corresponded to fungal genes involved in infection such as germination and penetration. The expression level of these genes returned to the same level as that of mock-inoculated plants by 24 h. But, by 72 h the gene expression diverged in resistant

genotype demonstrating that the defense response is regulated earlier in resistant genotype. Whereas, in susceptible genotype gene expression remained unaffected until 96 hai, the time period when fungal growth rapidly began. Genes involved in the biphasic response are associated with transcription, signal transduction and plant defenses, and are consistent with the stronger and more rapid induction of the defense genes typically seen in the hypersensitive response (HR). Choi et al. (2008) reported a microarray study of soybean accession PI200492, which contains *Rpp1*, after inoculation with two different isolates of *P. pachyrhizi* that resulted in susceptible or immune reactions. Up-regulation of peroxidases and lipoxygenase-like enzymes following rust inoculation was observed (Choi et al., 2008). A recent transcriptome analysis conducted by Soria-Guerra et al. (2010b) found that genes involved in the phenylpropanoid pathway were up-regulated early following rust infection of *G. tomentella*. Similarly, genes coding for proteins related to stress and defense responses such as glutathione-S-transferases, peroxidases, heat shock proteins, and lipoxygenases were also consistently up-regulated following infection until 72 hours. Tremblay et al. (2010) found many up-regulated genes associated with basic defense and down-regulated genes associated with many metabolic pathways in the rust-infected susceptible soybean palisade and mesophyll cells. However, little information can be inferred as to how these rust induced genes respond at the protein level on the basis of microarray studies, which examine how host genes respond to rust infection at the RNA level. Therefore, a proteomics-based investigation of host defenses is necessary to have a better understanding of how soybean responds to rust infection at the molecular level.

Proteomics is the study of the cellular proteome, defined as the set of proteins present in a biological unit (organism, organ, tissue, cell or organelle) at a specific developmental stage and under determined external biotic and abiotic conditions (Pérez-de-Luque et al., 2007). Use of proteomics offers several advantages such as understanding post-transcriptional modifications, and protein-protein interactions. The presence of large numbers of unknown genes in the plant genome and the lack of correlation between mRNA and protein levels (Gygi et al., 1999; Jansen et al., 2002; Watson et al., 2003) can also be addressed by the use of proteomics. Recently, Lee et al. (2009) successfully examined the host-pathogen interaction between bean and *Uromyces appendiculatus* using a proteomics approach. Similar studies have been done in barrel-clover and *Orobanche crenata* (Castillejo et al., 2009), wheat and *Puccinia triticina* (Rampitsch and Srinivasan, 2006), rice and *Magnaporthe grisea* (Kim et al., 2004), maize and *Aspergillus flavus* (Chen et al., 2004) and in soybean and *P. pachyrhizi* (Park et al., submitted to Planta).

Currently, the most common technique available for resolving thousands of proteins in a single run is two-dimensional gel electrophoresis (2-DGE), in which the separation in the first dimension is by isoelectric focusing and in the second dimension by molecular weight. The availability of wide range of pH gradients (3-12) allows the separation of highly acidic or basic proteins and provides an overview of total cellular extracts (Gorg et al., 1999). In contrast, narrow pH gradients of 1-1.5 pH units stretch protein patterns, allowing a more detailed investigation by providing enhanced resolution and aiding in the detection of minor components (Gorg et al., 2000; Wildgruber et al., 2000). Samples are often separated in multiple gels. The quantitative comparison of two 2-D gels requires

linear, uniform, and reproducible detection methods. Coomassie Brilliant Blue (CBB) dyes G and R stain fairly uniformly, but are limited by sensitivity (~100 ng) (Rabilloud, 2000). Silver staining provides low-nanogram range sensitivity and a good contrast. However, the sensitive silver staining methods may not be compatible with downstream mass spectrometric analysis of proteins of interest, and in one study only 77% of the silver-stained spots were shown to have a linear relationship with the total amount of protein present (Costa and Plomion, 1999). The more recently introduced SYPRO fluorescence dyes (Patton, 2000) allowed the detection of 1-10 ng of protein and the responses are linear over three orders of magnitude. This compares favorably with the CBB and silver staining for which the linear range is only about 40-fold, and which may vary from protein to protein (Merril, 1990; Steinberg et al., 1996). After staining, the scanned gel images can then be overlaid in order to identify differences in intensity or position of proteins from one gel to another. Often, variations between gels in spatial resolution and spot intensities make the overlaying of images and correct matching of proteins difficult, thus making it hard to distinguish biological variation from experimental variation. In other words, replicate 2-D gels are never identical, and despite the availability of specifically-designed image analysis programs, correct matching of all spots may be difficult.

Difference in-gel electrophoresis (DIGE) circumvents some of the above problems by enabling two samples covalently labeled with different fluorescent dyes of matching molecular masses to be run on the same gel (Ünlü et al., 1997). Cyanine-based dyes maintain the isoelectric point (p*I*) and mobility of labeled proteins, provide sensitivity equal to or better than silver staining, and improve comparative accuracy. As only 1 to

2% of all protein molecules are labeled by the dye, the method is compatible with mass spectrometric analysis of stained protein samples. The use of internal control of pooled samples makes DIGE a powerful and accurate tool in assessing protein changes across the experiment. In addition, the use of multi-color florescent dyes allows multiplexing of up to three separate protein samples on the same gel. This multiplexing capability of the DIGE methodology eliminates the major problem of gel to gel variation by incorporating the same internal standard on every gel, thereby increasing the accuracy and reproducibility (Lodha et al., 2013). In a typical 2D-DIGE experiment, proteins extracted from three different samples: healthy, diseased, and internal control (a pooled sample created by mixing equal amounts of the proteins extracted from the healthy and diseased samples), are each covalently labeled with cyanine fluorescent dye that has a different excitation and emission wavelength. Scanning the gel at the specific excitation wavelengths of each dye, using a fluorescence imager, allows visualization of the differentially labeled proteins. The images are then merged and analyzed using imaging software, which enables the differences in protein levels to be compared among different samples. DIGE eliminates any error related to gel misalignment and ensures an accurate quantification. 2D-DIGE has been successfully used to examine the changes of wheat xylase inhibitor protein families in response to infection with a Δ*Tri5* mutant of *Fusarium graminearum* (Dornez et al., 2010), responses of *Arabidopsis thaliana* to cold stress (Amme et al., 2006), and detection of inducible protein from *E. coli* (Ünlü et al., 1997).

Several recent studies have examined the soybean proteome in response to the symbiont *Bradyrhizobium japonicum* (Hempel et al., 2009; Wan et al., 2005), and to various stresses, including toxic metals (Sobkowiak and Deckert, 2006), salinity (Aghaaei et al.,

2009), flooding (Shi et al., 2008), and UV-B (Joseph et al., 2006; Xu et al., 2008). Herman et al. (2003) (Joseph et al., 2006) (Joseph et al., 2006) compared the allergens present in cultivars and wild type soybean and analyzed expression of allergens in transgenic soybean through proteomics. Protein profiles of soybean leaves, and root hairs, and during seed filling, also have been examined (Brechenmacher et al., 2009; Hajduch et al., 2005; Xu et al., 2006).

In a recent study, Park et al. (2010) compared protein profile changes in soybean cultivar 93M60 (Pioneer, Johnston, IA) in response to *P. pachyrhizi*. Forty protein spots that were differentially expressed 14 days after rust inoculation were identified, and 14 of them were sequenced using mass spectrometry. These proteins are involved in plant defense, stress, metabolism and other biological processes. Importantly, the pathogenesis related proteins, such as PR10, or defense related proteins, such as chalcone isomerase 1 (CHI1), were significantly induced at 10 hai and 6 dpi (days after inoculation), respectively. Thus, a proteomics approach can be effective in identifying key proteins mediating resistance of soybean against ASR. However, using varieties with different genetic backgrounds often poses difficulty in identifying the host proteins elicited by a particular pathogen. Therefore, selection of the right plant material in studying host-pathogen interactions is very important. In order to reduce the effects of the genetic background differences, near isogenic lines (NILs) that differ in resistance levels are ideal materials in proteomic studies for identification of the proteins directly involved in host resistance. The other advantages include accurate gel comparison and analysis to allow proteins differentially expressed at ratios as low as a two-fold between resistant and susceptible lines can be confidently identified (Chen et al., 2009). In addition, NILs have been utilized to identify

linkages between molecular markers and conventional phenotypic markers. NILs are important genetic stocks for investigating the function and regulation of single genes. They are typically developed by transferring a gene of interest into a different genetic background using multiple backcrosses to a recurrent parent. The genetic background of the NIL should then be nearly identical to that of the recurrent parent, except for the presence of a segment of DNA containing the introgressed gene. Genetic contributions to phenotypic differences in the responses of an NIL and its recurrent parent are therefore likely to be due largely to the selectively introgressed segment of DNA. The undescribed wheat genes responsible for partial leaf rust and stripe rust resistance were all studied through the use of near isogenic lines carrying known leaf rust resistance genes and their alleles originating from bread wheat, (Agarwal and Saini, 2009). Near-isogenic lines are also used for identifying resistance to stripe rust and powdery mildew, caused by *Puccinia striiformis* f. sp. *tritici* and *Blumeria graminis* f. sp. *tritici*, respectively, which are severe diseases in wheat (*Triticum aestivum*) (Liu et al., 2008).

Recombinant inbred lines are developed by crossing two inbred lines (parents) followed by repeated selfing of the generations derived after the cross between two parents, to create a new inbred line whose genome is a mosaic of the parental genomes (Broman, 2005). Sibling (or "sister") lines derived from the inbred are still segregating for a trait of interest and can also be used to study the effect of a gene that affects that trait, since like NILs, a large percentage of their genomes should be identical. Recently, some soybean sibling lines derived from recombinant partially inbred lines developed by D. Walker (USDA-ARS, Urbana, IL) showed differential reactions to infection by Florida soybean rust isolates (D. Walker, Personal communication). RIL population RN06-32-2 (32-2) was derived from a cross between Dillon [maturity group (MG) VI] and PI 605891A

(MG V) and population RN06-16-1 (16-1) was derived from a cross between breeding lines LG00-3372 (MG III) and PI 567104B (MG IX). The F1 derived from cross between respective parents was selfed until the F5 generation. In 2009, field screening was conducted in Quincy, FL with the F5 generation using Florida rust isolates. These sibling lines are derived from F5 plants, and they would therefore be expected to have genetic backgrounds that are approximately 93% similar. Although these lines are not ideal materials for identifying proteins associated with rust resistance, they should be useful for the tentative identification of candidate proteins using proteomics if they also show consistent differential expression following infection of the plants with Louisiana soybean rust isolates. Whether these infections induced proteins or differentially expressed between resistant and susceptible lines play any role in host resistance, still needs to be demonstrated.

CHAPTER 2: PROTEOMICS BASED STUDY OF SOYBEAN AND *PHAKOPSORA PACHYRHIZI* INTERACTION USING RECOMBINANT INBRED LINES

2.1 Introduction

Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* is one of the devastating diseases of soybean creating a major economic threat to the soybean industry. It was first described in Japan in 1902, and has since spread throughout the world in major growing areas (Ono et al., 1992). The first report of *P. pachyrhizi* in the United States was on a farm in Hawaii in 1994 (Killgore et al., 1994). *P. pachyrhizi* reached the continental United States. for the first time in November 2004 in Louisiana and several other southeastern United States (Schneider et al., 2005).

P. pachyrhizi is an aggressive foliar pathogen with a wide host range and is capable of overwintering on a number of alternative hosts, including kudzu (*Pueraria lobata*), leading to inoculum accumulation (Jurick et al., 2008; Park et al., 2008). The favorable environmental conditions, such as temperatures ranging between 15-26 °C and humidity as high as 80% promote ASR establishment (Levy, 2005). Yield losses caused by soybean rust ranged from 10-80% in South America and Asia under favorable environmental conditions (Bromfield, 1984; Kumudini et al., 2008; Ogle et al., 1979; Yang et al., 1990).

Currently, ASR is mainly controlled through fungicide applications because there are only a few resistant cultivars, for instance, INOX cultivars from Brazil marketed by TMG (Tropical Melhoramento & Genética) company. Cultural practices, such as enforcement of soybean free period, planting early maturing cultivars using wide row

spacing, adjusting soil fertility, are also effective in minimizing ASR losses (Rupe and Sconyers, 2008). Although the extensive use of the fungicides has reduced the yield losses of soybean to ASR in the U.S. and countries like Brazil, it is not cost effective in the long term. In addition, continuous increase in the use of fungicides has led to several serious concerns, such as fungicide resistance and toxicity to non-target aquatic organisms in the streams or ponds near the fields sprayed with the fungicides (Fernandez-Ortuño et al., 2008; Ochoa-Acuña et al., 2009). Fungicides also have shown toxic effect to mammalian cells based on laboratory assays (Daniel et al., 2007). For these reasons, improved host resistance of soybeans to ASR is considered the most viable alternative approach to fungicide applications.

At least six genes *Rpp1* to *Rpp6*, conferring single gene resistance to ASR have been reported (Bromfield and Hartwig, 1980; Cheng and Chan, 1968; Garcia et al., 2008; Hartwig and Bromfield, 1983; Hartwig, 1986; Hidayat and Somaatmadja, 1977; Hymowitz, 1980; Li et al., 2012; McLean and Byth, 1980b; Menkir et al., 2006; Singh and Thapliyal, 1977). However, these genes conferred resistance only against specific isolates of *P. pachyrhizi* (Bonde et al., 2006; Paul and Hartman, 2009; Pham et al., 2009). Part of the reason is that there is high genetic variation among *P. pachyrhizi* populations collected from different regions (Freire et al., 2008; Twizeyimana et al., 2011). Therefore, developing durable genetic resistance against ASR has been difficult.

Recently, several microarray studies were conducted to understand the host-pathogen interactions and to identify the genes involved in host defense response to ASR (Choi et al., 2008; De Mortel et al., 2007; Panthee et al., 2007; Soria-Guerra et al., 2010; Tremblay et al., 2010). These studies found that many up-regulated genes were

associated with basal defense and down-regulated genes were associated with many metabolic pathways in the rust-infected susceptible soybean leaf tissues. However, little information can be inferred from these studies as to how these rust-induced genes respond at the protein level. Therefore, a proteomics-based investigation of host defenses is necessary to have a better understanding of how soybean responds to rust infection at the molecular level.

In recent years, several studies have examined the soybean proteome in response to ASR (Cooper et al., 2011; Wang et al., 2012) and the proteome of ASR (Luster et al., 2010; Stone et al., 2012). Wang et al. (2012) used a resistant soybean cultivar to identify the differentially expressed proteins whereas Park et al. (2010) used a susceptible soybean cultivar. Many proteins, such as ascorbate peroxidase, glutathione transferase, chitinase, glycolate oxidase, heat shock protein, and iron superoxide dismutase, with a role in antioxidation and defense were found specifically up-regulated upon *P. pachyrhizi* inoculation in these studies. Recently, recombinant inbred lines (RIL) derived sibling lines, developed by D. Walker (USDA-ARS, Urbana, IL), showed differential responses to Florida rust isolates under field conditions. In this study, RIL derived sibling lines with similar genetic background were selected to use in a proteomic comparison in order to increase the chances of identifying soybean proteins playing a direct role in resistance to ASR and to better understand host-pathogen interactions.

The objectives of this study were to: 1) screen soybean RIL derived sibling lines for resistance to Louisiana rust isolates using both detached leaf assay and greenhouse inoculations 2) identify host and fungal proteins induced during compatible and incompatible interaction.

2.2 Materials and methods

2.2.1 Plant material

The soybean RIL derived sibling lines used in this study were developed by D. Walker (USDA-ARS, Urbana, IL). RIL population RN06-32-2 (32-2) was derived from a cross between Dillon [maturity group (MG) VI] and PI 605891A (MG V) and population RN06-16-1 (16-1) was derived from a cross between breeding lines LG00-3372 (MG III) and PI 567104B (MG IX). The F5:6 seeds from F5 plants were harvested, planted in the field in Quincy in 2009 and rated for resistance to ASR (D. Walker, personal communication). F5:6 seeds were obtained, and multiplied in the greenhouse, and then used as plant material for our experiments. Four sets of sibling lines from two RIL populations (Table 1) were used for screening against resistance to Louisiana rust isolates. The breeding line PI567104B and commercial variety AG6202 were included as resistant and susceptible controls, respectively.

2.2.2 Screening of sibling lines using a detached leaf assay against Louisiana rust isolates

Soybean sibling lines of 8-a, 8-b, 8-c, 94-a, 94-b, and 94-c of population 32-2, 15-b, 15-c, 16-b, and 16-c of population 16-1, and AG6202 were grown in four 20-cm diameter plastic pots (four seeds per pot) per line in a greenhouse. Soybean rust (*P. pachyrhizi*) urediniospores were collected from infected soybean leaves at the Central Station, of the Louisiana State University Agricultural Center, Baton Rouge, in 2008 and were stored at -80 ºC. A rust spore suspension was prepared with 0.01% Tween-20 and the concentration of 3×10^4 spores/ml was determined using a hemocytometer. Six

soybean leaves were collected randomly from each line at R1 stage and were inoculated with 200 µl of rust suspension on the upper (adaxial) surface. After rust inoculation, the leaves were transferred to a Petri dish lined with water-soaked Whatman filter paper, and were incubated at 25 ºC in 16 h of light and 8 h of darkness. The disease severity of each line was rated 14 dpi by examining six leaves for development of rust symptoms from each line grown in four replications. On each leaf, six 1-cm^2 areas were marked randomly on the lower (abaxial) side of a leaf and were later observed for the lesions and uredinia formation. The count was recorded for each of the six areas on each leaf and the average number of lesions or uredinia per 6 cm^2 total inspected area on a leaf was calculated. On the basis of the average number of lesions and uredinia, a RI score was calculated for each line. The relative resistance of each line was scored using the Rust Index (RI) score previously developed to evaluate Florida rust isolates (Walker et al., 2011). The RI score, is the product of a rust severity rating (1 = no lesions and 5 = high density of lesions, similar to that observed on susceptible check plants inoculated at the same time) and the sporulation rating (1 = no sporulating uredinia, 5 = high density of sporulating uredinia). Lines with RI score of 1 were ranked as I (Immune, with no lesions and no sporulation visible on the sampled leaves), RI = 2-9 as R (Resistant, with low to moderate numbers of lesions and low sporulation), RI = 12-16 as M (Moderately resistant, with a moderate level of disease, but substantially less than the most susceptible lines), and RI = 20-25 as S (Susceptible, with a high level of density of heavily sporulating lesions), respectively.

2.2.3 Screening of soybean sibling lines under greenhouse conditions

For greenhouse *in planta* inoculations of each line, 64 plants in 16 pots at R1 stage were inoculated by spraying 200 ml of sterile distilled water containing 0.01% Tween-20 and a urediniospore suspension at a concentration of 3 x 10^4 spores/ml. Furthermore, each pot was kept dark in a bio-hazard bag with 10-15 wet paper towels to maintain a high humidity at 25 ºC. As mock-inoculated controls, another 64 plants in 16 pots were sprayed with 200 ml of sterile distilled water containing 0.01% Tween-20, and were otherwise treated in the same manner as the inoculated plants. All 32 pots were incubated in a dark room at 25 ºC for two days and were placed back in the greenhouse on the third day. Rust infection was observed from 7 dpi, and it was more prominent at 10 dpi. Rust pustules were visible on the abaxial side of the leaves of inoculated plants and showed variation in the response to rust for different lines. The scoring for rust infection was done at 14 dpi. RI scoring as above was used to rank each of the lines in a greenhouse.

2.2.4 Time-course experiment for DIGE proteomics

Identifying host and fungal proteins induced during rust infection as well as when and at what level they are expressed, is a first step in understanding molecular host-pathogen interactions. For this purpose, a time-course experiment was conducted. Soybean sibling lines 8-a (resistant) and 8-c (susceptible) of population RN06-32-2 (Table 1) were selected for the proteomics study. Soybean plants were inoculated with the rust spores as described in the greenhouse screening. Leaf samples collected immediately after inoculation was labeled as 0 hrs. Further, sample collection was done at 0 h, 10 h, 1 d, 2

d, 3 d, 5 d, 8 d, 10 d, 12 d and 14 d after inoculation. The leaf samples were stored at -80 °C until further use for DIGE proteomics.

2.2.5 Protein extraction

The leaf tissue was ground in liquid nitrogen and proteins were extracted using a phenol method (Hurkman and Tanaka, 1986). Protein pellets were air-dried for 10 min and stored at -30 °C until further use in electrophoresis.

2.2.6 Two dimensional electrophoresis

Isoelectric focusing (IEF). The protein samples were centrifuged at 14,000 rpm for 10 min at room temperature (RT) and supernatant was transferred to a fresh 1.5 ml microfuge tube and protein concentration was determined using a protein assay buffer (Bio-Rad) (Bradford, 1976). Cy dye labeling for each protein sample was done according to the manufacturer's directions (GE Healthcare, Piscataway, NJ) with minor modification where control or infected samples were labeled with a ratio of 200 pmol Cy2, Cy3 or Cy5 protein minimal labeling dye for each 60 µg of protein samples. For the gel normalization, an internal control was prepared by pooling an equal protein quantity from each of the samples. The Cy2, Cy3, and Cy5 labeled samples were pooled into a microcentrifuge tube, mixed with equal volume of 2X sample buffer [2 M thiourea, 7 M urea, 2% w/v 3-[(3- cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS)] and the final volume was adjusted to 340 µl with rehydration buffer [2 M thiourea, 7 M urea, 2% w/v CHAPS, 2% v/v 3-10 nonlinear (NL) immobiline pH gradient (IPG) buffer, 20 mM dithiotheritol (DTT)] before being added to the 18 cm 3-10 NL IPG strips for overnight rehydration. IEF was performed at 20 °C for 8 hrs under the

following conditions: 90 min at 500 V, 90 min at 1,000 V, and 5 hr at 8,000 V. The focused strips were first equilibrated immediately for 20 min in 7 ml of sodium dodecyl sulfate (SDS) equilibration buffer (75 mM Tris-HCl pH 8.8, 6 M urea, 30% w/v glycerol, 2% w/v SDS) with 0.5% w/v DTT per strip and this was followed by a second equilibration of 20 min in 7 ml of SDS equilibration buffer with 4.5% w/v iodoacetamide (IAA).

SDS-PAGE. The equilibrated IPG strips were embedded in 1% agarose overlay solution on top of a 12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) analytical gel [235 mm (width) x 190 mm (length) x 1.5 mm (thickness)] for the second dimension (Ettan DALT*twelve* large vertical system, GE Healthcare) (Laemmli, 1970). Electrophoresis was carried out at 22 °C at a constant voltage of 110 V for 1800 Vhrs.

2.2.7 Image acquisition and trypsin digestion

The CyDye-labeled analytical gels were scanned with Typhoon™ 9410 (GE Healthcare) variable mode imager at a resolution of 100 μm, using the appropriate filters for the excitation/emission wavelengths of each dye (i.e., Cy2-488/520 nm; Cy3-532/580 nm; and Cy5-633/670 nm). The voltages of the photomultiplier tube (PMT) were adjusted for a maximum image quality with minimal signal saturation. The images were checked for saturation during the acquisition process using Progenesis Samespots gel analysis software (Nonlinear Dynamics, www.nonlinear.com). Scanned gel images were analyzed in all possible combinations to find differentially up- or down-regulated protein spots between inoculated and control leaf samples from resistant and susceptible lines. The criteria for selecting spots for sequencing are based upon their up- or down-regulation in

the resistant line 8-a, at different time points or all the time points and the fold change ($p \geq 0.05$) compared to the susceptible line 8-c upon infection. These selected protein spots were excised from 2 to 3 CBB G-250 preparative gels (Candiano et al., 2004) and subjected to in-gel digestion with trypsin (Proteomics grade trypsin, Sigma, Cat # T6567) as previously described (Shevchenko et al., 2007). The digested peptides were subjected to either matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS) as described below.

2.2.8 Protein identification using LC-MS/MS

Twenty nine protein spots were sequenced using LC-MS/MS at the Pennington Biomedical Center Proteomics core facility. The digested peptide fragments were extracted with 2% (v/v) acetonitrile and 1% (v/v) formic acid and transferred to a 96-well plate for analysis. The peptides from each digested spot were separated by a capillary LC system coupled to a nanospray quadrupole time-of-flight (Q-TOF) tandem mass spectrometer (Waters Corp). Briefly, the peptides were injected onto a 75 µm C_{18} reverse phase capillary column (Dionex) and separated using a gradient of 3 to 40% acetonitrile during a 30 min run. The MS was operated in a data-dependent acquisition mode, in which a full survey of the parental ions was followed by three MS/MS scans using normalized collision energy. The instrument was operated in positive ion mode, with an electrospray voltage of 3.5 kV, sample cone voltage of 40 V and extraction cone voltage of 1.5 V. The peaklist (pkl) files were generated using ProteinLynx Global Server 2.2.5 (PLGS 2.2.5, Waters Corp.) with default parameters. Tandem mass spectra were searched against the SwissProt database using the PLGS 2.2.5 software (Waters Corp.) with the

following settings: one missed tryptic cleavage; precursor-ion mass tolerance, 200 ppm; fragment-ion mass tolerance, 0.1 Da and fixed carbamidomethylation of cysteine residues. Methionine oxidation of proteins was allowed as a variable modification in the database search query in PLGS, and auto modification query was selected to identify peptides with further post-translational modifications in PLGS. The top ranking hits (PLGS scores between 8-13) were further evaluated using molecular weight, p*I*, and % sequence coverage to help confirm protein identities.

2.2.9 Protein identification using MALDI-TOF MS

Eight protein spots were sequenced using MALDI-TOF MS at the Pennington Biomedical Center Proteomics core facility. The peptide solution was analyzed using a Micromass® MALDI-TOF (reflectron) mass spectrometer. The pkl files were generated using PLGSwith default parameters. The resulting pkl file containing peptide mass fingerprinting data were queried against the protein database in NCBInr using MASCOT software (http://www.matrixscience.com) with the following search parameters: *Viridiplantae* (green plant), trypsin, up to one missed cleavage, carbamidomethylation of cysteine and oxidation of methionine, peptide tolerance 1.2 Da, mass value MH+ and monoisotopic.

2.2.10 Statistical analysis

For the detached leaf assay and greenhouse screening, experimental records were subjected to analysis of variance (ANOVA) using the mixed procedure (PROC MIXED) of SAS (version 9.1, SAS Institute, Cary, NC) was performed on data from number of lesions and number of uredinia for all sibling lines tested in both detached leaf assay and

greenhouse study. Means were compared by Tukey's Honestly Significant Difference (HSD) at $p < 0.05$ with Kramer adjustment for unbalanced design (Kramer, 1956).

For proteomics data, gels from a minimum of three biological replicates were included in gel analysis using Progenesis Samespots v2.0 (Nonlinear dynamics). The protein profiles from infected lines 8-a were compared to 8-c followed by ASR inoculation. The protein spots that showed ≥ 1.1 folds up- or down-regulation in line 8-a and are statistically significant ($p < 0.05$) were selected for further analysis. Protein profiles from leaf collected at 10 hrs, 2 d, 5 d, 8 d and 12 d were analyzed to find common spots which showed ≥ 1.1 fold differences in both infected resistant line compared to the infected susceptible line with the $p \leq 0.05$.

2.3 Results

2.3.1 Detached leaf screening

Four sets of RIL derived sibling lines from two different populations that showed differential responses to the ASR population in Quincy, Florida in 2009 according to RI score ranking (Table 1), were screened using the detached leaf assay for their resistance to soybean rust isolates from Louisiana. The results are summarized in Table 1. The representative appearance of the soybean leaves 14 dpi for each line is shown in Figure 1. Among the ten soybean lines, five lines showed significant difference ($p < 0.05$) in lesion type/size and number of uredinia compared to their corresponding sibling line when screened against the Louisiana rust isolates using the detached leaf assay. The lines 15-b (Fig. 1A) and 16-c (Fig. 1D) of population 16-1 and the lines 8-a (Fig. 1H), 8-b (Fig. 1I) and 94-c (Fig. 1G) of population 32-2, which all showed immune reaction to Florida rust isolates, exhibited resistant response against Louisiana rust isolates, with very few reddish brown lesions and no sporulation similar to the resistant control PI567104B (Table 1),

whereas the other five lines showed the same response to Louisiana rust isolates as to Florida rust isolates. Lines 15-c (Fig. 1B) and 16-b (Fig. 1C) of population 16-1 showed a moderately resistant response with appearance of reddish brown lesions as well as sporulation. These lines produced significantly ($p < 0.05$) fewer rust pustules than the susceptible control AG6202 after rust inoculation. Lines 94-a (Fig. 1E), 8-c (Fig. 1J) of population 32-2 and 94-b (Fig. 1F) of population 32-2 showed susceptible and resistant responses, respectively, against Louisiana rust isolates. The eruption of the sporulating tan lesions on the susceptible sibling lines (8-c and 94-a) were much faster (as early as 7 dpi) and more profuse than the moderately resistant sibling lines (sporulation was observed at 10 dpi).

2.3.2 Greenhouse screening

When these RIL lines were screened for rust resistance in the greenhouse, significant differences ($p < 0.05$) in the disease parameters were also observed among the soybean sibling lines. The lines showed similar resistant or susceptible responses to ASR in greenhouse inoculations as in the detached leaf assay (Table 1). The number of reddish brown lesions, tan lesions, and the amount of sporulation were relatively less when screened under greenhouse conditions (Table 1). The representative appearance of the soybean leaves 14 d after ASR inoculation for each line is shown in Figure 2.

2.3.3 Time-course proteome analysis of sibling lines after ASR infection

After confirming the differential responses of RIL sibling lines to Louisiana rust isolates, one of the four sets of the sibling lines (the resistant line 8-a and susceptible line 8-c from population 32-2) was selected for a time-course analysis of proteome profile changes during rust infection. The profiles of leaf proteins extracted from ASR infected and

Table 1. Summary of the screening of sibling lines from two different inbred populations using a detached leaf assay

			Detached leaf assay		Greenhouse inoculation		
Population	**Response to Florida isolates in 2009 [u]**	**Response to Louisiana isolates [u,v]**	**Lesions [w, x]**	**Uredinia [x,y]**	**Lesions [w, x]**	**Uredinia [x,y]**	**Rust Index (RI) score [z]**
RN06-16-1 15-b	I	R *	8.6 b	ND	3.9 b	ND	3
16-1 15-c	M	M	ND	6.9 c	ND	2.9 c	12
16-1 16-b	M	M	ND	10.6 c	ND	3.6 bc	12
16-1 16-c	I	R *	3.6 d	ND	1.8 c	ND	2
RN06-32-2 94-a	S	S	ND	19.0 ab	ND	3.9 b	25
32-2 94-b	R	R	6.0 b	ND	2.1 c	ND	3
32-2 94-c	I	R *	6.3 c	ND	2.0 c	ND	3
32-2 8-a	I	R *	4.0 d	ND	1.9 c	ND	2
32-2 8-b	I	R *	4.2 d	ND	1.9 c	ND	2
32-2 8-c	S	S	ND	15.4 b	ND	3.9 b	20
Susceptible Control	S	S	ND	20.6 a	ND	7.8 a	25
Resistant Control	R	R	14.9 a	ND	7.50 a	ND	2

[u] I = Immune, R = Resistant, M = Moderately Resistant, and S = Susceptible
[v] * indicates different response to Louisiana isolates compared to Florida isolates
[w] Mean number of reddish brown lesions per cm^2 leaf area
[x] Means in the same column followed by the same letter are not significantly different at $\alpha = 0.05$ according to the Tukey-Kramer test
[y] Mean number of uredinia per cm^2 leaf area
[z] product of the rust severity rating (1-5) and the sporulation rating (1-5); 1 = Immune, 2-9 = Resistant, 12-16 = Moderately resistant, 20-25 = Susceptible
ND = Not determined
RI score = for detached leaf assay

mock-inoculated control plants at 10 h, 2 d, 5 d, 8 d and 14 d revealed approximately 1100±100 protein spots for both 8-a and 8-b lines when resolved using 18 cm, pH 3-10 NL IPG strips for the first dimension and 12.5% SDS-PAGE for the second dimension (Figure 3). Reproducible protein patterns were observed in at least three out of four biological replicates that we compared. Protein profile differences between the resistant and susceptible line under non infection (mock inoculation) conditions were also compared.

The comparison identified 100 differentially expressed spots. Seventy three spots were significantly up-regulated and 27 spots were significantly down-regulated in the resistant line 8-a compared to the susceptible line 8-c. Among the 73 differentially expressed spots, 37 spots were selected for sequencing based on the criteria of their up- or down-regulation in the resistant line 8-a, at different time points or all the time points and the fold change ($p \geq 0.05$) compared to the susceptible line 8-c upon infection.. Figure 3 illustrates the differentially expressed protein spots between resistant line 8-a and susceptible line 8-c after rust inoculation in the superimposed two dimensional protein profile of the two lines. In this gel picture, the spots which were differentially expressed are either white (up-regulated in 8-a) or yellow in color (down-regulated in 8-a) (Figure 3). It appears that the difference in the number and the diversity of the proteins differentially expressed between resistant and susceptible lines is genotype dependent (Table 2, Table 3 and Figure 4). For examples, spots S3, S11, S30, S31, S32, S33, S37 and S47 were up-regulated in the resistant line whereas S13 and S21 were up-regulated in the susceptible line at 10 h after ASR inoculation. Spots S15, S26, S28, S34, and S35; S11, S17, S20, and S35; S3, S20, S23 and S35; and S13, S18, S19, S21, S29 and S35 were up-regulated

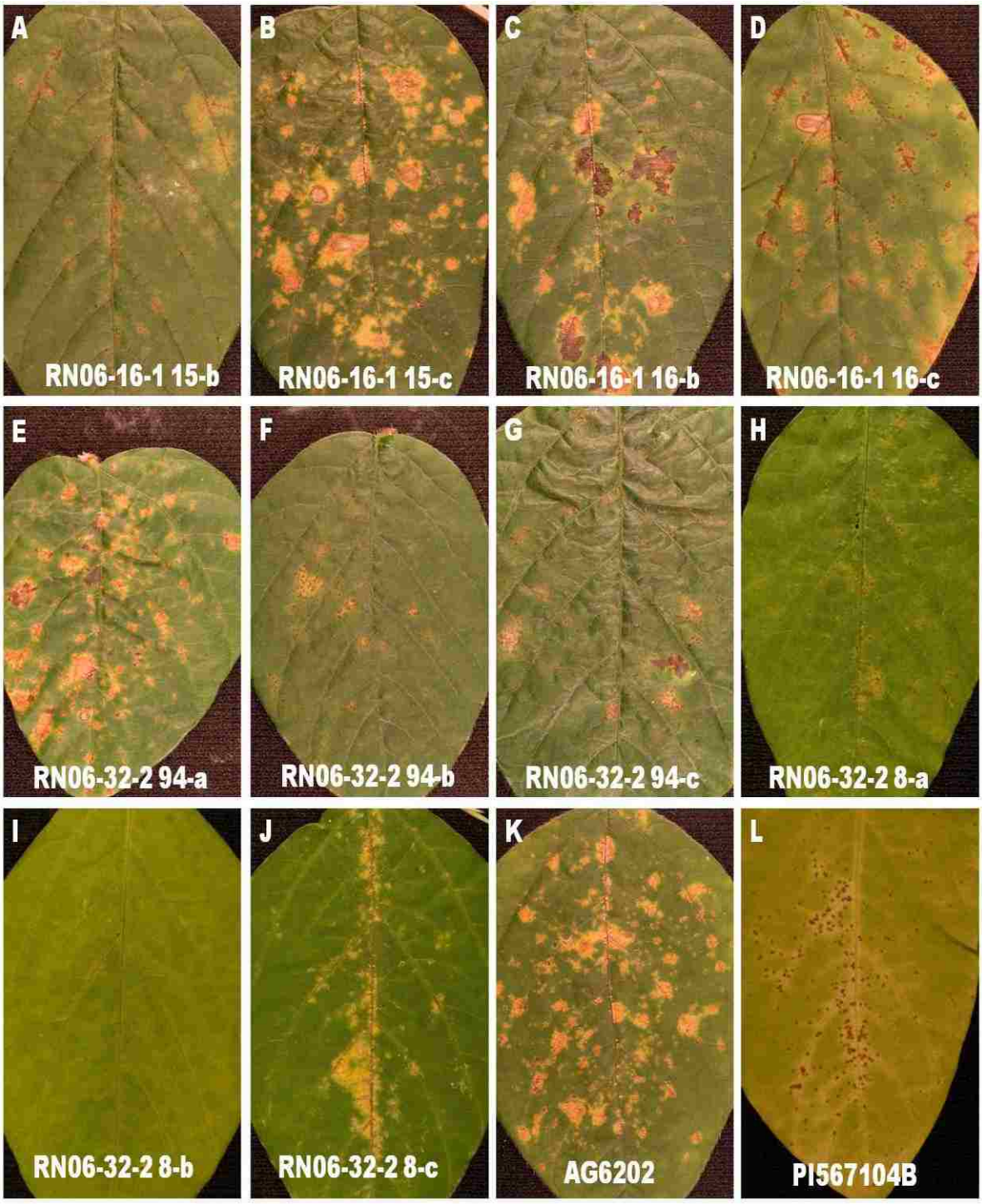

Figure 1. Symptoms at 14 dai in detached leaf assay on sibling lines from two different inbred populations inoculated with Louisiana rust isolates. A to D represent sibling lines belonging to population RN06 16-1; E to J represent sibling lines belonging to population RN06-32-2; K, AG6202, susceptible control; L, PI567104B, resistant control.

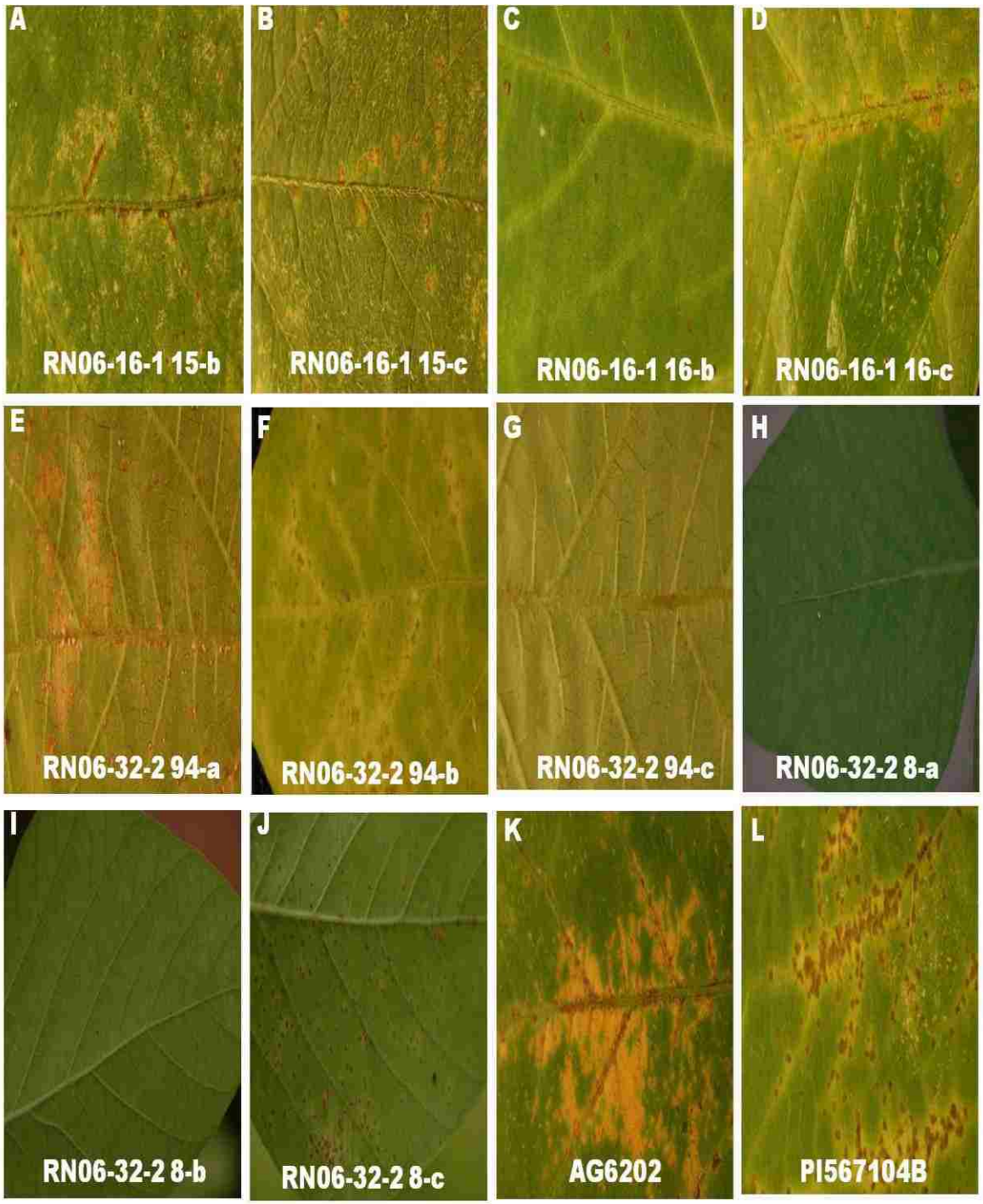

Figure 2. Symptoms at 14 dai in detached leaf assay on sibling lines from two different inbred populations inoculated with Louisiana rust isolates. A to D represent sibling lines belonging to population RN06 16-1; E to J represent sibling lines belonging to population RN06-32-2; K, AG6202, susceptible control; L, PI567104B, resistant control.

in the resistant line at 2, 5, 8, and 12 dpi, respectively (Table 2 and Table 3). Few of the protein spots were up-regulated in the susceptible line, such as S2 and S21 at 5 dpi, S3 at 8 dpi, S13, S17 and S21 at 12 dpi (Table 2 and Table 3). The spots which were up-regulated in the resistant line compared to susceptible line after ASR infection at all the time points are S1, S5, S7, S8, S9, S10, S12, S22, S25 and S36 (Table 2 and Table 3). Examples of some of the proteins that showed significant up-regulation in response to ASR inoculation are shown in Figure 5.

2.3.4 Identification of differentially expressed proteins

Thirty up-regulated spots and seven down-regulated spots in resistant line 8-a compared to the susceptible line 8-b in response to rust inoculation (Table 2 and Table 3) were sequenced using LC-MS/MS spectrometry and MALDI-TOF/ MS analysis. These protein spots were identified based on peptide mass fingerprinting for MALDI-TOF and MS/MS ion search for LC-MS/MS using the mascot search engine (Perkins et al., 1999). The biological functions and the cellular localization of these proteins were obtained based on information from Uniprot (www.uniprot.org/) and from Plant-Ploc (http://www.csbio.sjtu.edu.cn/ bioinf/plant/#), respectively (Table 4). A majority (70%) of the identified proteins (S5, S6, S9, S11 to S28, S32, S33, S35, S36 and S37) are putatively located in the chloroplast, followed by the cytoplasm (S1, S2, S7, S29 and S31) (13.5%), the plasma membrane (S3, S4 and S30) and the mitochondria (S8, S10 and S34) (8.1%) (Figure 6 and Table 4). Twenty seven percent of the identified proteins are involved in photosynthesis (S5, S6, S7, S8, S9, S11 and S14) according to the biological

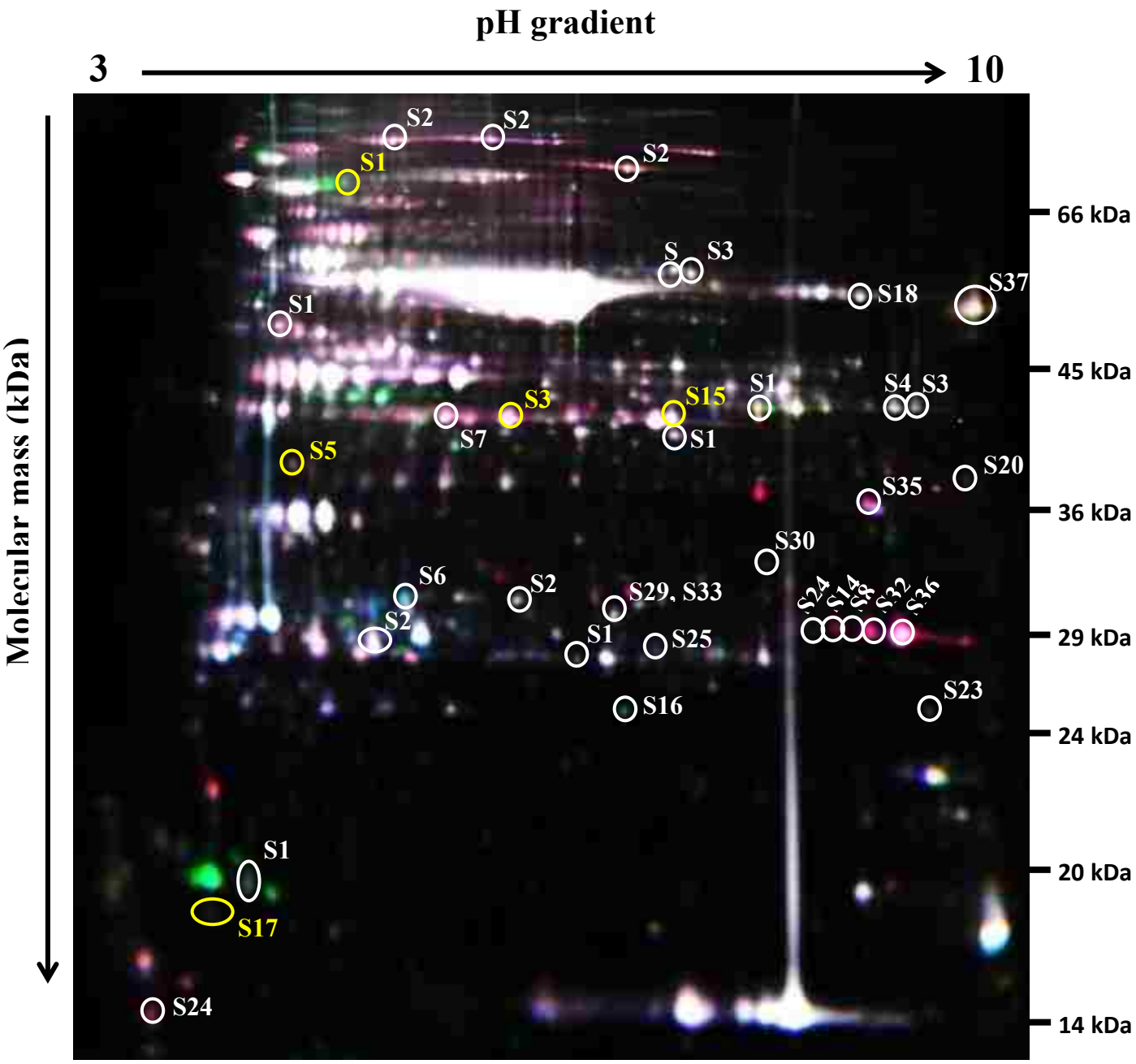

Figure 3. Protein spots differentially expressed in proteome of resistant line 8-a and susceptible line 8-c, in response to infection by ASR. Spots up-regulated in line 8-a are shown in white font and down-regulated are shown in yellow font. MW=molecular weight; pH gradient 3-10 NL

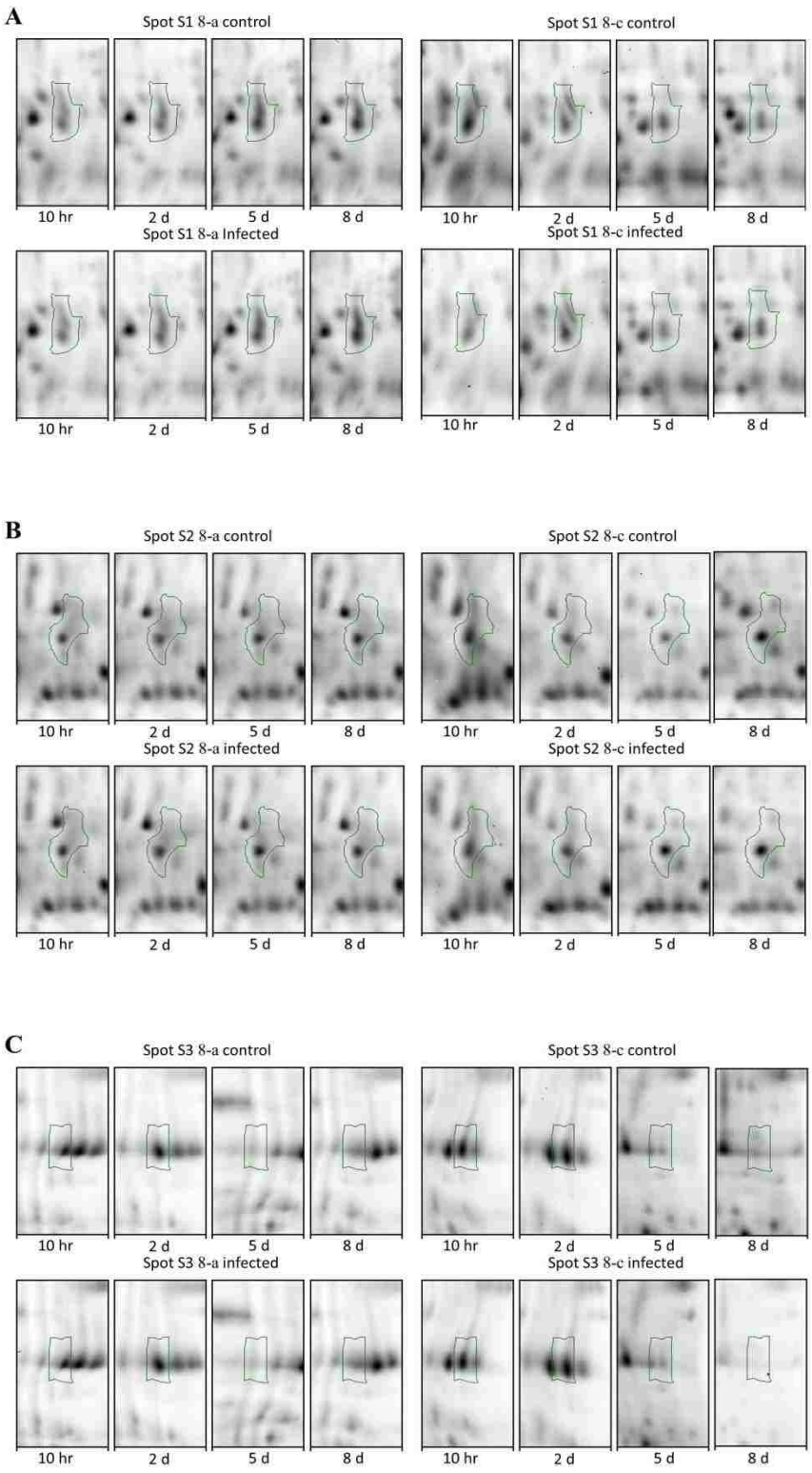

Figure 4. Gel sub-sections of few of the spots under control and inoculated conditions A. Spot S1 B. Spot S2; C. Spot S3.

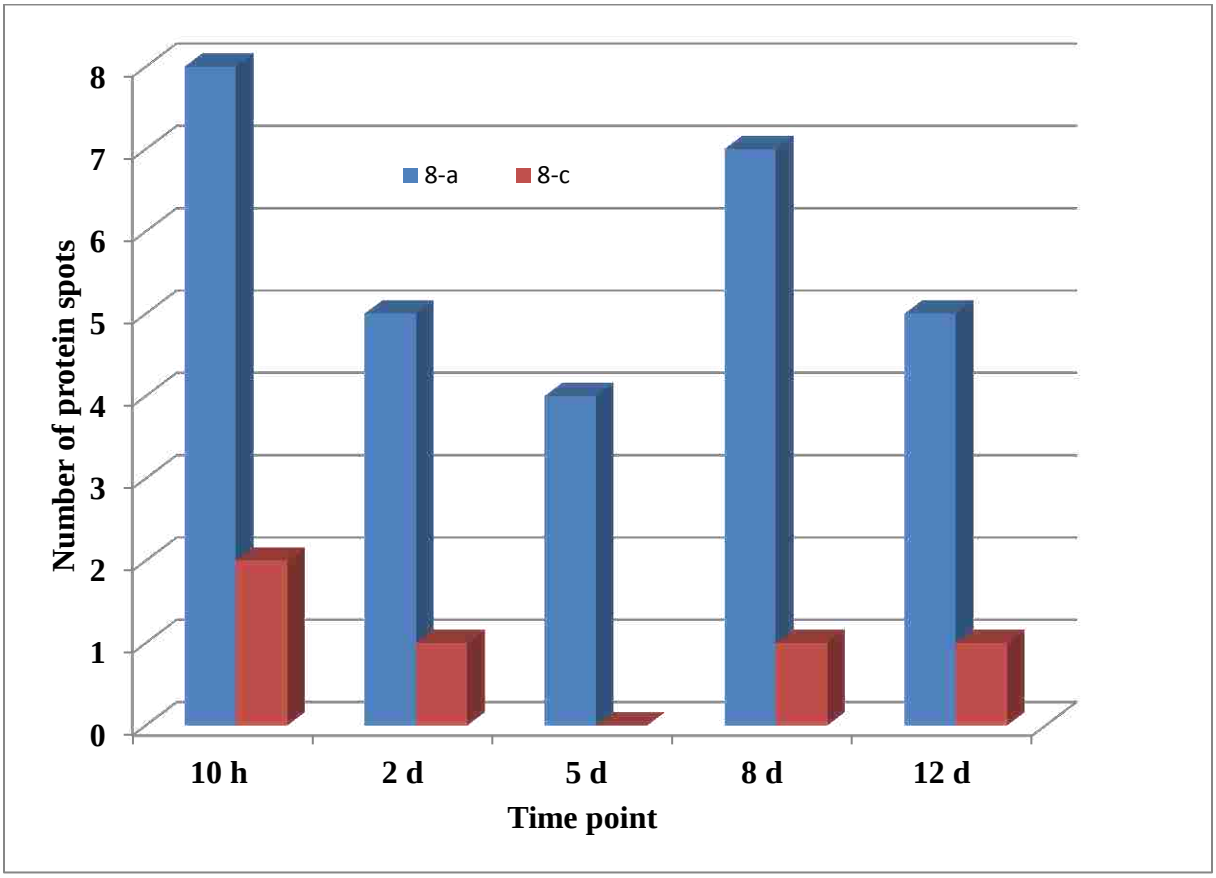

Figure 5. Comparison of number of up-regulated spots in between infected resistant line 8-a and infected susceptible line 8-c at 10 h, 2 d, 5 d, 8 d and 12 dpi.

function, followed by seed storage protein and other cellular processes (S22 to S26, S32, S33, S36, S10, S12, S13, S15, S16, S18, and S29 to S31) (49%), plant defense (S1, S2, S3, S4 and S17) (13.5%), protein translation (S10, S19, S20 and S21) (10.8%) and unknown (S27 and S28) (5.4%) (Figure 7 and Table 4).

Table 2. Protein identifications and properties of spots differentially expressed in resistant line RN06-32-2 8-a and susceptible line RN06-32-2 8-c in response to ASR infection

Spot number[a]	Time point	Fold change	Putative protein	NCBI accession number	No. of matching peptides[b]	Peptide sequence[c]	Peptide Mascot score[d]	Peptide *m/z*[d]	Protein Mascot score[d]	% Sequence coverage[e]	Theoretical p*I*[d]	Theoretical MW (Da)[d]
S1 ↓	5 d	2.3	Stress-induced protein SAM22	NP_001236038	3	alVTDADNvIPK aieAYLLAHPdyn sveNVEgnGGPGTiKK	65 56 44	628.37 745.39 529.29	175	25	4.69	16762
S2	All	1.2	Ascorbate peroxidase 2	AAB01221	9	tGGPFGTIK eGLLQLPSDK gsdHLRdVFGK syPTVSADYQk gkSYPTVSADyqK aLLSDPVFRPlVEK syPTVSaDYQkAvEk hpselahGannGlDIavr yasdeDAFFADYAeAHqk	26 68 60 76 63 75 48 17 72	439.22 550.28 615.79 629.77 722.34 528.62 562.60 624.29 693.27	506	38	5.65	27123
S3	12 d	4.6	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like	NP_001241302	5	nfEGLDLGk aiALTVDTpr vPVFLDGgvR vPVFLdGgvrr iAVQSGaAGIIVSNhgar	26 50 61 43 93	496.74 528.79 529.78 405.56 574.29	272	12	9.16	40768
S4	12 d	3.9	Peroxisomal glycolate oxidase	NP_001238412	6	wlqTITK nvVAQLVR nfEGLDLGK lPVFLDGgvr lPvFLDGgvrR iaiqSgaaGIIVSNHGar	18 45 40 50 30 65	445.25 449.77 496.75 536.80 614.85 578.97	248	14	9.01	40781
S5 ↓	All	1.5	Ribulose bisphosphate carboxylase large chain	YP_538747	4	DTDLLAAFR YGRPLLGCTIKPK AVYECLR TFQGPPHGIQVER	NA	511.24 501.59 455.70 489.23	106	NA	6.00	53033
S6	5 d	1.1	Ribulose bisphosphate carboxylase small chain 1, chloroplastic, precursor	P00865	2	iIGFDNvR taYPNGfiR	51 17	467.24 519.25	68	9	8.87	20060
S7	All	2.0	Malate	NP_001236661	3	VLVTGAAGQIGYALVPMIAR MELVDAAFPLLK	NA	672.68 673.86	172	NA	6.32	35527

(Table 2 continued)

Spot number[a]	Time point	Fold change	Putative protein	NCBI accession number	No. of matching peptides[b]	Peptide sequence[c]	Peptide Mascot score[d]	Peptide *m/z*[d]	Protein Mascot score[d]	% Sequence coverage[e]	Theoretical p*I*[d]	Theoretical MW (Da)[d]
			dehydrogenase			VLVVANPANTNALILK		825.47				
S8	5 d	1.8	Serine hydroxymethyltransferase 5	NP_001237509	9	fAQALSER	43	461.23	437	17	8.83	57342
						qFPTIGFEK	45	533.76				
						gfVEEDFVK	39	535.25				
						liVAGASAYar	64	546.29				
						vAEFFDAAvk	61	548.76				
						vLEAVHIAANK	73	582.81				
						elLYDYEDk	47	594.25				
						sslPDeAvYdk	16	612.27				
						ayQEQVLSNsfk	51	707.32				
S9	8 d	3.1	Ribulose bisphosphate carboxylase large chain	YP_538747	11	ipTAYIK	21	403.25	487	25	6.00	53033
						aVYECLR	40	455.74				
						alrLEdLR	26	493.31				
						dTDILAAFR	64	511.29				
						lTYYTPdYetk	44	697.35				
						lEDLrIPtAYIK	43	477.96				
						lSGgdHVHAgtvVgk	55	478.60				
						tfqGPPHGIQver	55	489.27				
						yGrpllGctiKPk	54	501.64				
						ghylNatAGtCEemMKr	40	667.64				
						ggldftkddenvNSQPFMr	45	729.35				
S10 ↓	12 d	1.3	Heat shock 70 kDa protein, mitochondrial-like, predicted	XP_003543129	7	iAGLDVqr	19	435.76	317	10	5.68	72383
						hLNITLtR	42	484.28				
						eiEDAVSDLr	57	573.76				
						ttPSVVAFNqk	56	596.31				
						vqqVVSeIFGk	52	617.81				
						nsADTSIYSIek	29	618.76				
						qAVTNPtNTLFGTk	66	746.37				
S11	12 d	1.3	Probable fructose-bisphosphate aldolase 2, chloroplastic-like, predicted	XP_003537836	9	eAAWGLaR	52	437.22	580	20	8.24	42925
						aAQDALLFR	85	502.76				
						aGSYADElVk	45	526.75				
						saAYYqQGar	44	557.75				
						aSPqTVADYTLK	78	647.31				
						laSIGLENTEANR	86	694.33				
						tvVSIPNGPSaLAVK	40	726.90				
						dkASPQTVADYTLK	87	768.86				
						rlasiGlENTEANr	62	515.25				
S12	All	1.6	Chaperone protein ClpC, chloroplastic-like isoform 1	XP_003523172	7	aqISTLVEK	36	495.26	413	8	6.16	102490
						tAIAEGlAqr	52	515.27				
						aiDLIDEAGsR	51	580.27				
						aIMLAQEEar	70	580.28				
						lQHAQLPEEAR	84	431.21				

(Table 2 continued)

Spot number[a]	Time point	Fold change	Putative protein	NCBI accession number	No. of matching peptides[b]	Peptide sequence[c]	Peptide Mascot score[d]	Peptide *m/z*[d]	Protein Mascot score[d]	% Sequence coverage[e]	Theoretical *pI*[d]	Theoretical MW (Da)[d]
						lAEEGkLDPvVGr	75	461.57				
						vlENLGADPtnir	54	706.35				
S13	8 d	1.2	Aconitate hydratase, cytoplasmic-like	XP_003540302	2	llNgeVGPk	17	463.75	68	1	8.12	107174
						lYVFDAAqR	53	541.77				
S14	8 d	1.4	Oxygen-evolving enhancer protein 2, chloroplastic	XP_003551942	16	hQLITATVK	49	505.82	1923	58	7.68	28417
						eVEYPGQVlr	44	595.34				
						fVestAssfsVA	31	616.32				
						qYYSLTVLtr	52	622.36				
						lSIPSKWNpsk	43	628.82				
						hQLITATVKDGk	71	437.59				
						rfvestassFSva	81	694.36				
						tnTDFLSYNGngfk	72	789.35				
						wnPSKEVEYPGQvlr	78	901.46				
						tADGdEGgKHQLITATVK	135	920.95				
						tntdflsyYnGnGfKLSIPSK	33	735.39				
						yEDnFDSTSNVAVMVTATDKk	108	1168.05				
						sitDYgsPeEFlSkVDYLLgk						
						kSiTDYGspeEFLSQvDYLlGk	58	1181.10				
						qaffgqtdaeggfdsnavatanilESSTPvV	117	830.46				
						Dgk	30	1148.56				
						qaffgqtdaeggfdsnavatanilesstPvvDG						
						kQYYSltVLtr	18	1167.82				
S15 ↓	All	1.6	Probable aldo-keto reductase 1	NP_001236007	4	elGIGIVPYsplgr	60	735.97	214	15	6.14	38457
						iknldqNIGALAVK	61	499.65				
						yiGLSEASPdTirR	79	526.62				
						diEEEIVPlCR	39	686.85				
S16	All	1.4	Probable aldo-keto reductase 1	NP_001236007	6	lSEKDLR	50	430.78	174	19	6.14	38457
						lGTQGFEVSK	26	533.32				
						nlDQNIGALAVK	55	628.40				
						dieeEIVPlcr	11	686.88				
						elGigiVPYsplgr	15	735.96				
						yigLsEASPdTirR	17	526.65				
S17 ↓	All	1.4	Superoxide dismutase [Fe], chloroplastic	NP_001238486	10	tyVENLk	15	433.76	329	51	5.6	27881
						qvVGtELDGK	27	523.32				
						sleEIIVTSYnK	52	698.41				
						lvSWDAVSSrleqak	23	563.67				
						aaaatqfGSgWAwLAYr	36	914.01				
						fdgeNvaNppSpDEdnklvVLK	24	799.77				
						qvvgtekdgkSlEEIIVTSynk	40	808.14				

(Table 2 continued)

Spot number[a]	Time point	Fold change	Putative protein	NCBI accession number	No. of matching peptides[b]	Peptide sequence[c]	Peptide Mascot score[d]	Peptide *m/z*[d]	Protein Mascot score[d]	% Sequence coverage[e]	Theoretical p*I*[d]	Theoretical MW (Da)[d]
						rpdyisvfmdKLVSWDAVSsr	46	829.79				
						fldefkaaaatqfGSgwAWLAYr	44	869.48				
						felkpppypLngLEpvmsqqtlEFHWgk	21	825.46				
S18	All	1.4	Uncharacterized protein LOC100801140	XP_003537975	15	nlAPNKAVvK	41	527.84	1192	34	8.61	55510
						aaSLAQEAQEK	39	573.32				
						aGVPELGSAQELAr	93	699.38				
						sqPLTIQEFlqk	52	716.43				
						vIETDVKYtFIK	49	485.97				
						vDELFSPiPedGr	47	737.41				
						vDELFSPiPedgrr	52	543.97				
						laTQYKIISNeQAK	95	803.98				
						dpSTVFVAGATGqAGiR	89	823.97				
						lnaVQSSFdNADTiAK	84	847.47				
						vfgglfkqetiYVDDD	18	923.50				
						rlnavQSsFDNaDTIAK	86	925.52				
						iaslVADVFSNTEVAeNK	86	954.04				
						IASLVADVFSNTEVAENKVVK	121	745.11				
						lnAVQSSFDNADtiAKAIGnagk						
						aasLAQEAQeKAEAGGASVENLL	107	769.10				
						NK	135	833.80				
S19	8 d	1.1	30S ribosomal protein 2, chloroplastic-like, predicted; Chalcone isomerase A	XP_003531427; ABI54176	5; 3	faFVTMk	20	422.22	182; 112	14; 13	8.73; 6.32	26772; 23232
						vYVGNLAK	27	432.24				
						lYVGNiPR	40	466.25				
						rLYVGnIPr	54	544.31				
						tvEDATAVIEK;	44;	588.30;				
						diISGPfEk	22	503.25				
						tYFLGGAGeR	48	535.75				
						ilPLAGAEYSK	44	581.31				
S20	All	1.3	30S ribosomal protein S5, chloroplastic-like	XP_003529335	3	akEVIAAVQk	42	528.86	190	12	9.18	32004
						qlGSNNALNNar	81	636.37				
						ySTFPHRADGdYGAAK	68	585.97				
S21	8 d	1.6	50S ribosomal protein L10, chloroplastic-like	XP_003549555	7	elVTVLk	26	401.25	295	27	9.43	25805
						nLESLPtR	29	465.25				
						fYGPDEVK	34	477.73				
						nleeqqgvAQ	14	558.75				
						spASALVGTLQSPar	72	727.89				
						leDNdFTGAVFEGk	78	771.35				
						kledndftGavFEGk	41	835.38				
S22	10 hrs	3.2	31 kDa protein,	AAA33938	6	mAVTEANlk	36	488.74	269	24	8.64	28877

(Table 2 continued)

Spot number[a]	Time point	Fold change	Putative protein	NCBI accession number	No. of matching peptides[b]	Peptide sequence[c]	Peptide Mascot score[d]	Peptide *m/z*[d]	Protein Mascot score[d]	% Sequence coverage[e]	Theoretical p*I*[d]	Theoretical MW (Da)[d]
			partial			lAVEAHNIR	61	511.77				
						dyINGEQFR	34	571.74				
						nyNKLLSLGFK	40	648.85				
						tvNQQAFFYASer	68	780.84				
						gdaPALPeTlk	30	556.28				
S23	5 d	1.5	Stem 31 kDa glycoprotein, precursor	P10742	5	iiFLSGR	34	403.24	273	19	8.59	32862
						gnAPALPETLK	52	555.80				
						tvNQQAYFYar	55	680.81				
						tLDKQAVTEANLK	83	715.88				
						dPqDPSTPnavSYk	49	759.84				
S24	All	1.8	Stem 31 kDa glycoprotein, precursor	P10742	4	iIFLSgR	29	403.25	232	14	8.59	32862
						gnAPALPeTLK	53	555.80				
						tvNQQAYFYar	58	680.83				
						dPQDPSTPnAVsyk	92	759.84				
S25	All	4.2	Stem 31 kDa glycoprotein	NP_001241536	5	mAVTEANLK	63	496.74	236	19	6.72	29433
						lAVEAHNIR	59	511.77				
						gdaPALPetLK	44	571.27				
						dyINGEQFR	22	578.26				
						tiPEECVEPtK	57	651.80				
S26	All	1.5	Stem 31 kDa glycoprotein	NP_001241536	8	mAVTEANLK	55	496.77	422	31	6.72	29433
						lAVEAHNIr	68	511.81				
						gdaPALPeTlk	33	556.32				
						dyINGEQFR	32	571.29				
						tiPEECVEPtK	55	651.84				
						dPHLITPnalsyk	37	734.92				
						ylDKMAVTEANLk	82	756.41				
						tvNqQAFFYASer	61	780.90				
S27	5 d & 8 d	2.2	Uncharacterized protein At4g01050, chloroplastic-like	XP_003528797	5	KLLFAeDR	55	496.27	307	12	5.96	46429
						qVGSPDVGGlK	49	528.78				
						qlDEFLNtK	30	554.77				
						adAVAPEVNsvPK	61	648.83				
						lGaDGNAQLLDIr	115	678.35				
S28	10 hrs & 12 d	1.4 & 2.0	Unknown	ACU23213	5	aGvFTVGDK	42	447.22	228	16	5.41	38120
						aSEEFDPLLk	73	574.77				
						niDSGGeLTek	44	581.75				
						irtdPdLanar	24	621.31				
						dggtYIDPIAPggsadk	49	804.34				
S29	All	1.2	Uncharacterized protein	NP_001235654	3	gkDIVELIAagr	56	621.35	132	39	4.36	11439
						latvpsggggaVaaaPGggaAAAAPaae	47	853.75				

(Table 2 continued)

Spot number [a]	Time point	Fold change	Putative protein	NCBI accession number	No. of matching peptides [b]	Peptide sequence [c]	Peptide Mascot score [d]	Peptide *m/z* [d]	Protein Mascot score [d]	% Sequence coverage [e]	Theoretical *pI* [d]	Theoretical MW (Da) [d]
			LOC100499761			ak latvPsggggavavaaaPggGaaaaAPaaeakk	29	896.44				

↓ Indicates spots down-regulated in line 8-a

[a] Spot identification number (Fig. 3)

[b] Number of identified unique peptides by Mascot MS/MS ion search

[c] lower case letters indicate no confidence based on Mascot MS/MS ion search

[d] Obtained from Mascot, Protein score is -10*Log(P), where P is the probability that the observed match is a random event

[e] The values indicate the percentage of sequence coverage of identified peptides

Table 3. Protein identifications using MALDI-TOF and properties of spots differentially expressed in resistant line RN06-32-2 8-a and susceptible line RN06-32-2 8-c in response to ASR infection

Spot number[a]	**Putative protein**	**NCBI accession number**	**No. of matching peptides[b]**	**Mascot score[c]**	**Theoritical pI[c]**	**Theoritical MW[c] (Da)**
S30, S37	Gamma glutamyl hydrolase precursor	NP_001235549	22, 19	163, 133	6	37653
S31	dihydrolipoyl dehydrogenase	XP_003550821	24	52	6.7	53276
S32, S36	Stem 28 kDa glycoprotein	NP_001238459	19, 26	145, 183	8.8	29046
S33	Stem 31 kDa glycoprotein	NP_001241536	25	165	6.8	29261
S34 ↓	Serine hydroxymethyltransf-erase 5	NP_001237509	38	96	8.6	57110
S35	Ribulose bisphosphate carboxylase large chain	YP_538747	32	134	5.9	52576

↓Indicates spots down-regulated in line 8-a
a Spot identification number (Fig. 3)
b Number of identified unique peptides by Mascot PMF
c Obtained from Mascot, score is -10*Log(P), where P is the probability that the observed match is a random event

2.3.5 Possible involvement of the differentially expressed proteins in soybean resistance to rust

The peptide sequence of spot S1 identified it as stress induced protein SAM22 (NP_001236038) (Crowell et al., 1992; Kleine-Tebbe et al., 2002), which was down-regulated at 5 dpi. It showed high sequence similarity (78% to 100%) to other protein from *G. max* such as PR10-like protein, uncharacterized protein (NP_001236562), and soybean allergen Gly M4 (Figure 8). Spot S2 was

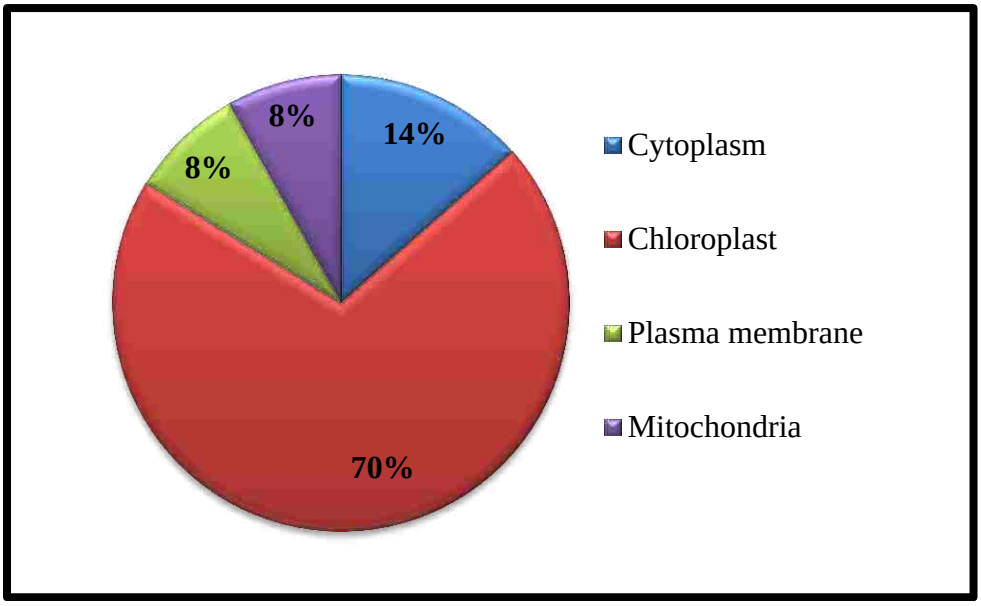

Figure 6. Sub-cellular localization of identified proteins in soybean differentially expressed in response to ASR infection

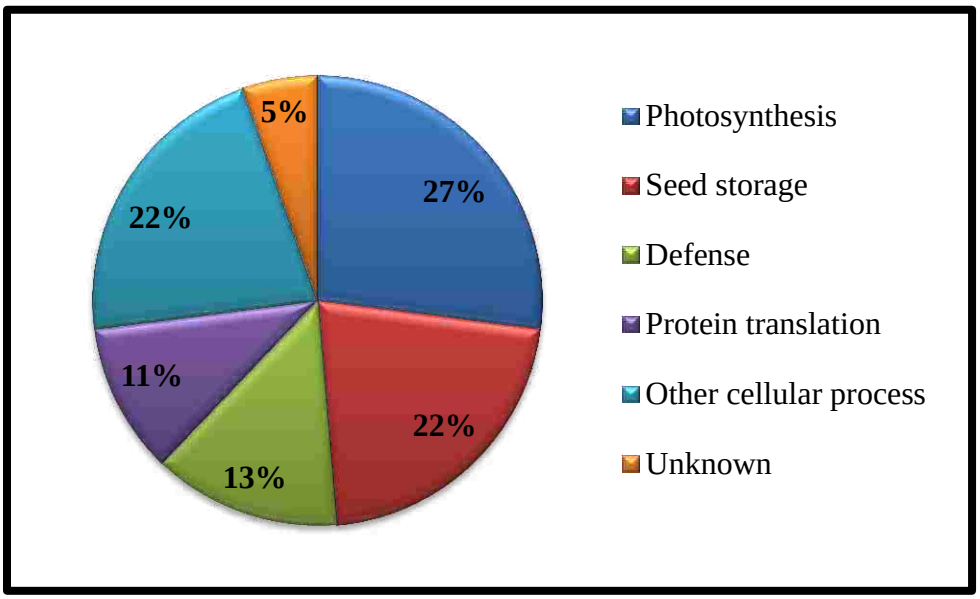

Figure 7. Biological function of identified proteins in soybean differentially expressed in response to ASR infection

identified as ascorbate peroxidase 2 (AAB01221) (Caldwell et al., 1997; Chatfield and Dalton, 1993; Dalton et al., 1986; Dalton et al., 1996; Shi et al., 2008) from *G. max,* Spots S3 and S4 were identified as peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like (NP_001241302 and NP_001238412). The spots S5, S6, S9 andS35 were identified as ribulose-1, 5 bisphosphate carboxylase/oxygenase (RuBisCO) (YP_538747, P00865 and YP_538747). Several other protein spots were identified as stem 31kDa glycoprotein, such as S22, S23, S24, S25, S26, S32, S33 and S36 (AAA33938, P10742, P10742, NP_001241536 NP_001241536, NP_001238459 and NP_001241536). Spot S8 (NP_001237509) and S34 (NP_001237509) were identified as serine hydroxymethyltransferase 5 (SHMT). Spot S7 was identified as malate dehydrogenase (MDH) (NP_001236661). Spots S10 and S12 were identified as heat shock 70 protein (XP_003543129) and Chaperone protein (XP_003523172), respectively. Spot S13 was identified as aconitate hydratase (XP_003540302), which catalyzes the formation of isocitrate from citrate during the second step of the citric acid cycle (Kaneda et al., 2007). Spot S14 had a sequence identical to oxygen-evolving enhancer protein, which is one of the most important proteins for oxygen evolution in Photosystem II (PSII). Spots S15 (NP_001236007) and S16 (NP_001236007) were identified as aldoketo reductase I (AKR). Spot S17 (NP_001238486) was identified as superoxide dismutase [Fe] (SOD). Spots S18 (XP_003537975), S27 (XP_003528797), S28 (ACU23213) and S29 (NP_001235654) had sequence identical as proteins of unknown function. Spots S19 and S20 were identified as 30S ribosomal protein 2 (XP_003531427) and 30S ribosomal protein S5 (XP_003529335), respectively. Spot S21 was identified as 50S ribosomal protein L10 (XP_003549555). These proteins are potentially involved in protein synthesis (Carter et al., 2000). Spots S30 and S37 (NP_001235549) were

Table 4. Putative functions and subcellular localization of the identified proteins

Spot number	Putative protein	Hypothetical function	Subcellular localization
Defense Response			
S1	Stress-induced protein SAM22	Pathogenesis related protein, Plant defense response, Response to biotic stimulus	Cytoplasm
S2	Ascorbate peroxidase 2	Response to oxidative stress, Peroxidase activity	Cytoplasm
S3	Peroxisomal (S)-2-hydroxy-acid oxidase GLO1-like	Oxidoreductase activity	Plasma membrane
S4	Peroxisomal glycolate oxidase	Oxidoreductase activity	Plasma membrane
Photosynthesis and metabolism			
S5	Ribulose bisphosphate carboxylase large chain	Photosynthesis	Chloroplast
S6	Ribulose bisphosphate carboxylase small chain 1, chloroplastic, precursor	Photosynthesis	Chloroplast
S7	Malate dehydrogenase	Photosynthesis	Cytoplasm
S8	Serine hydroxymethyltransferase 5	Photosynthesis	Mitochondrion
S9	Ribulose bisphosphate carboxylase large chain	Photosynthesis	Chloroplast
S10	Heat shock 70 kDa protein, mitochondrial-like, predicted	Protein folding, ATP binding	Mitochondrion
S11	Probable fructose-bisphosphate aldolase 2, chloroplastic-like, predicted	Photosynthesis	Chloroplast
S12	Chaperone protein ClpC, chloroplastic-like isoform 1	Protein metabolic process, ATP binding, Nucleoside-triphosphatase activity	Chloroplast
S13	Aconitate hydratase, cytoplasmic-like	4 iron, 4 sulfur cluster binding	Chloroplast
S14	Oxygen-evolving enhancer protein 2, chloroplastic	Photosynthesis, Calcium ion binding	Chloroplast
S15	Probable aldo-keto reductase 1	Oxidoreductase activity	Chloroplast

(Table 4 continued)

Spot number	Putative protein	Hypothetical function	Subcellular localization
S16	Probable aldo-keto reductase 1	Oxidoreductase activity	Chloroplast
S17	Superoxide dismutase [Fe], chloroplastic	Superoxide metabolic process	Chloroplast
S18	uncharacterized protein LOC100801140	Nucleotide binding	Chloroplast
S19	30S ribosomal protein 2, chloroplastic-like, predicted	Translation, RNA binding	Chloroplast
S20	30S ribosomal protein S5, chloroplastic-like	Translation, RNA binding	Chloroplast
S21	50S ribosomal protein L10, chloroplastic-like	Translation, RNA binding	Chloroplast
Seed Storage Proteins			
S22	31 kDa protein, partial	Seed storage protein, Acid phosphatase activity	Chloroplast
S23	Stem 31 kDa glycoprotein, precursor	Seed storage protein, Acid phosphatase activity	Chloroplast
S24	Stem 31 kDa glycoprotein, precursor	Seed storage protein, Acid phosphatase activity	Chloroplast
S25	Stem 31 kDa glycoprotein	Seed storage protein, Acid phosphatase activity	Chloroplast
S26	Stem 31 kDa glycoprotein	Seed storage protein, Acid phosphatase activity	Chloroplast
	Unknown		
S27	Uncharacterized protein At4g01050, chloroplastic-like	Unknown	Chloroplast
S28	Unknown	Unknown	Chloroplast
S29	Uncharacterized protein LOC100499761	Translational elongation, Structural constituent of ribosome	Cytoplasm
S30	Gamma glutamyl hydrolase precursor	Glutamine metabolic process, Gamma-glutamyl-peptidase activity	Plasma membrane
S31	Dihydrolipoyl dehydrogenase	Cell redox homeostatis, Dihydrolipoyl dehydrogenase activity, Flavin adenine dinucleotide binding	Cytoplasm

(Table 4 continued)

Spot number	Putative protein	Hypothetical function	Subcellular localization
S32, S36	Stem 28 kDa glycoprotein	Seed storage protein, Acid phosphatase activity	Chloroplast
S33	Stem 31 kDa glycoprotein	Seed storage protein, Acid phosphatase activity	Chloroplast
S34	Serine hydroxymethyltransferase 5	Photosynthesis	Mitochondrion
S35, S37	Ribulose bisphosphate carboxylase large chain	Photosynthesis	Chloroplast

identified as gamma glutamyl hydrolase precursor. Spot S31 (XP_003550821) was identified as dihydrolipoyl dehydrogenase (DD). These findings validate the differential expression of proteins between resistant and susceptible lines upon inoculation with rust detected by proteomics study.

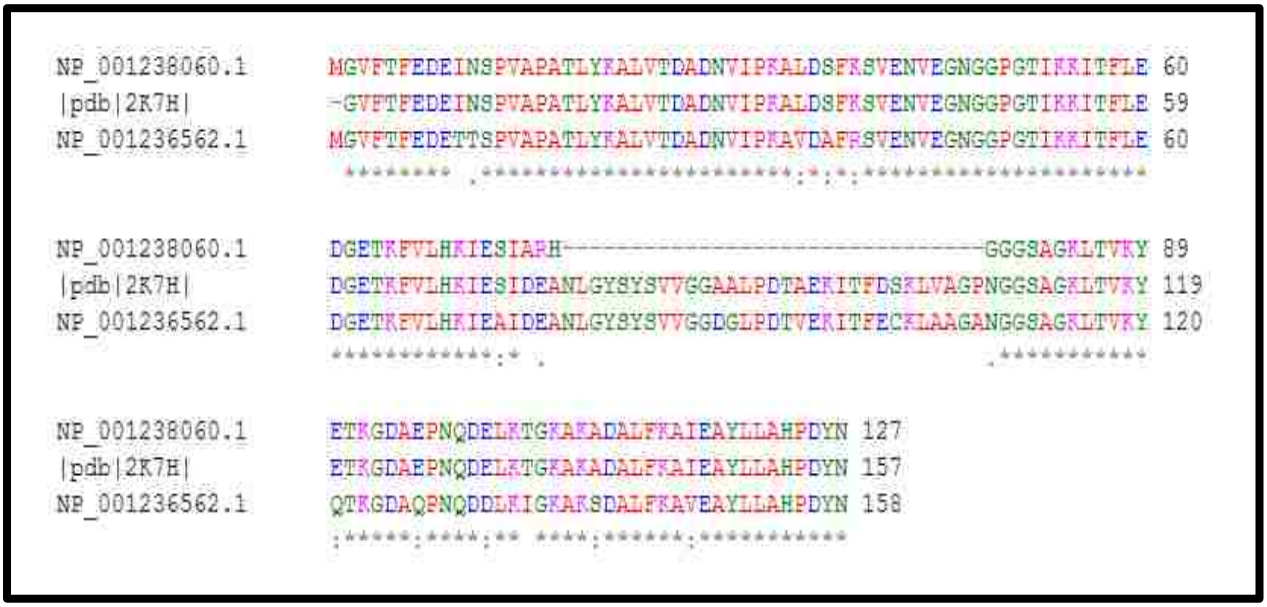

Figure 8. Multiple sequence alignment of PR10-like protein (NP_001238060) and soybean allergen Gly M4 (PDB: 2K7H_A) and uncharacterized protein (NP_001236562)

2.4 Discussion

2.4.1 Detached leaf assay and greenhouse screening

In the present study, 10 sibling lines from two RIL populations were screened with Louisiana rust isolates using two different methods. We observed the consistent differential responses between the soybean sibling lines under both screening conditions. The detached leaf assay appeared to be more rapid and reliable compared to the greenhouse inoculation method. Due to its more controlled and uniform environmental conditions and the ability to evaluate different populations and/or different host plants all year round, the detached leaf assay has been widely used to evaluate host plant resistance against various pathogens, such as *Phytophthora infestans*, *Stagonospora nodorum* and the diseases Fusarium head blight and powdery mildew (Benedikz et al., 1981; Brown and Wolfe, 1990; Diamond and Cooke, 1999; Vleeshouwers et al., 1999). The key to reproducible results in detached leaf assays is to keep the detached leaves green and healthy. This can be achieved by amending the agar medium with different levels of cytokinin, and gibberellic acid for retarding the chlorosis and senescence (Burdon and Marshall, 1981; Twizeyimana et al., 2007).

In addition, the detached leaf assay often produces more severe rust disease symptoms (number of RB lesions, uredinia, etc.) compared to greenhouse inoculations. This could be the result of a reduced level of resistance expression in the detached leaf assay compared to inoculation on intact plants. Similar results have been observed in the study by Vleeshouwers et al. (1999), which demonstrated that the integrity of the plant is necessary for complete resistance response.

Five of the ten sibling lines (lines 15-c and 16-b of population 16-1, and lines 94-a, 94-b and 8-c of population 32-2) evaluated against Louisiana rust isolates in the present study showed the same responses as to Florida rust isolates according to their RI scores. Whereas the other five lines (15-b and 16-c of population 16-1 and the lines 8-a, 8-b and 94-c of population 32-2), which had a RI score of one and was considered as an immune response when evaluated under field natural inoculation in Florida, produced sporadic pustules/lesions without sporulation when evaluated against Louisiana rust isolates. These responses were considered as a resistant reaction rather than an immune reaction due to the resulting rust index scores of 2 to 9. This minor difference could be attributed to the differences in virulence between the two rust isolates (Twizeyimana et al., 2007). The difference in inoculum concentration might be another factor. Compared to natural inoculations with Florida population, the uniform and high inoculum concentration (disease pressure), and the continuous availability of favorable environmental conditions with Louisiana rust isolates may have partially overcome the quantitative resistance.

2.4.2 Proteomics

To better understand the differences at the molecular level between the sibling lines that show differential rust resistance and to identify potential candidate proteins/genes involved in rust disease resistance, RILs 8-a (resistant) and 8-c (susceptible) from population RN06-32-2 were further studied through proteomics. Most of the differentially expressed proteins identified in this study have complex changes during the entire period of rust infection, possibly due to the complicated nature of the signaling pathway in the defense mechanism upon pathogen recognition (Berger et al., 2007; Bolton et al., 2008). Several recent microarray studies reported the biphasical expression of many soybean genes in responding to rust infection (van de Mortel

et al., 2007) and found that the susceptible soybean lines were able to induce the same set of genes, but at a lower level or at a later time. In addition, the proteomic study of soybean during rust infection by Park et al. (2013) noticed that the expression of rust infection induced proteins is regulated both at the transcription and post transcription levels.

2.4.3 Rust infection reduces photosynthesis

P. pachyrhizi is a biotrophic pathogen which primarily infects above ground tissue typically leaves causing rust and mainly affects the photosynthesis process. Therefore, there are many obvious reasons that explain why the primary metabolism of the plant was disturbed after the pathogen attack. Rust reduces the leaf surface area for photosynthesis due to formation of reddish brown or tan lesions and chlorosis. As a result, plant will be under a lot of pressure to meet the requirement of energy demand to induce the defense against the pathogen. The redistribution and diversion of energy causes reduction or increase in the plant primary metabolism and contributes to fight against the pathogenic infection (Berger et al., 2007).

In this study, it is very interesting to note that approximately 60% of the sequenced proteins are involved in the photosynthesis process or metabolism (Table 4 and Figure7) and most of them are up-regulated in the incompatible interaction. To begin with, RuBisCO (S5, S6, S9 and S35) was identified from different locations on the 2D gels (Figure 3).

RuBisCO is one of the key enzymes involved in the CO_2 fixation and conversion into energy rich molecules such as glucose in the Calvin-Benson cycle. Previous studies (Berger et al., 2007; Bonfig et al., 2006; Chou et al., 2000; Doehlemann et al., 2009) show that the rate of photosynthesis decreases upon pathogen attack, wounding or herbivore attack, in both

compatible and incompatible interactions. In this study, the large subunit of RuBisCO (S9) and precursor for the small subunit (S6) were up-regulated in the resistant line compared to the susceptible line, following rust inoculation. It was also interesting to see two low molecular weight spots (S5 and S35) corresponding to the large subunit of RuBisCO, which are possibly the degradation products (Bernardo et al., 2012).

Oxygen-evolving enhancer (OEE1) (S14) is a key component of PSII. 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). A study done in 2002 (Abbink et al., 2002) showed that silencing of a gene encoding a protein component of the oxygen-evolving complex of PSII enhances virus replication in plants. The up-regulation of this protein could help the plant in generating more reactive oxygen species (ROS) for the hypersensitive reaction (HR) during the rust infection process.

2.4.4 Rust affects respiration, photorespiration and induces defense

Respiration pathways such as glycolysis, tricarboxylic acid (TCA) cycle and mitochondrial electron transport are known to be stimulated during resistance response (Bolton, 2009). The various interconnected pathways of plant respiration are meant to generate energy that can be used for plant defense upon pathogen attack. Aconitate hydratase (S13), catalyzes the formation of isocitrate from citrate during the second step of the citric acid cycle, itsup-regulation probably enhances energy production (Kaneda et al., 2007). This protein was also up-regulated in the incompatible interaction of rice and *Magnaporthe grisea* (Lu et al., 2004). Malate dehydrogenase

(MDH) (S7) is one of the important enzymes playing a crucial role in many metabolic processes including the tricarboxylic acid cycle, amino acid synthesis, gluconeogenesis and facilitation of exchange of metabolites between cytoplasm and subcellular organelles (Musrati et al., 1998). It has been shown that the reduced activity of the isoform of MDH has enhanced the photosynthesis and plant growth (Nunes-Nesi et al., 2005). In this study, MDH was down-regulated at all the time points by 2 folds in the incompatible interaction of *P. pachyrhizi* and soybean.

A number of reports have shown the correlation between photorespiration and disease resistance (Bolton et al., 2008; Okinaka et al., 2002; Sørhagen et al., 2013; Taler et al., 2004). Serine hydroxymethyltransferase 5 (SHMT) (S34) which was down-regulated, functions in the photorespiratory pathway in catalyzing the reversible conversion of serine and glycine with tetrahydrofolate serving as the one-carbon carrier. SHMT also catalyzes the folate-independent retroaldol cleavage of allothreonine and 3-phenylserine and the irreversible conversion of 5,10-methenyltetrahydrofolate to 5-formyltetrahydrofolate (Szebenyi et al., 2004). This reaction provides the largest part of the one-carbon units available to the cell. A recessive mutation, *shmt1-1* in *Arabidopsis* resulted in over-production of ROS. *shmt1-1* mutants also showed slightly decreased expression of *PR1*, *PR2*, and *PR5* genes compared with control plants in response to *Psuedomonas syringae* pv. *tomato* DC3000 *avrRPM1* (Moreno et al., 2005; Sørhagen et al., 2013). Also, it has been revealed that the *SHMT* gene is down-regulated under stress condition. In this study, stress induced protein SAM22 (S1) (PR10-like protein) was down-regulated at 5 dpi concomitant with the down-regulation of SHMT. Heat shock 70 protein (S10) is down-regulated at 12 d time point by 1.3 folds. The heat shock proteins (Hsps) are the proteins

which cope with stress-induced denaturation of other proteins (Feder and Hofmann, 1999). The role of Hsps in R protein mediated hypersensitive response and non-host resistance to pathogens in *Arabidopsis thaliana* and *Nicotiana benthamiana* have been reported (Kanzaki et al., 2003). The down-regulation of SHMT in our study may indicate that the biotic stress decreased the expression of SHMT and its low levels resulted in over production of ROS by the up-regulation of GLO causing a destructive effect and compromised the resistance by lowering the expression of PR10 protein and Hsps.

Rust resistant and susceptible soybean lines showed differential expression of antioxidant and defense related proteins. For instance, GLO (S3 and S4), a key enzyme in photorespiration, catalyzing the oxidation of glycolate to glyoxalate, was up-regulated. GLO has been shown to be an essential component of non-host defense response to *Pseudomonas syringae* in *Arabidopsis* and for tobacco *Pto/AvrPto*-mediated defense response and alternative source for the production of H_2O_2 during both gene-for-gene and non-host resistance responses (Rojas et al., 2012). To remove the excess H_2O_2 generated by GLO and OEE, one of the important ROS scavenging enzymes, Ascorbate peroxidase (APX) (S2), is up-regulated at all the time points (Sørhagen et al., 2013).

Superoxide dismutase [Fe] (S17) was down-regulated by 1.4 fold at all time points. Superoxide dismutases (SODs) are metal-containing enzymes that catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide. This enzyme has been found in all aerobic organisms examined where it plays a major role in the defense against toxic-reduced oxygen species, which are generated as byproducts of many biological oxidations (Asada, 1999; Bowler et al., 1994). The regulation of SOD in this study is contrary to the previous studies (Bolton, 2009; Mittler,

2002), wherein excessive ROS production is scavenged by enzymes such as APX, catalase (CAT) and SOD which have enhances expression upon pathogen attack. In this study, it is possible that the down regulation of SOD is compensated by the enhanced expression of APX.

Glycoprotein (S22, S24 to S26, S32, S33 and S36) was up-regulated by 1.5 to 4.2 fold in the incompatible interaction at all time points. These glycoproteins are shown to be involved in host resistance apart from their role in plant metabolism or growth and development (Beber et al., 2002; Jakobek and Lindgren, 2002; Liu et al., 2005). In this study, the up-regulation of glycoproteins during all the time points of rust infection process indicates that they may serve as a temporary storage pool for amino acids. Furthermore, they will be utilized in the energy generating pathways by shuttling of the amino acid metabolism, for the defense mechanism. Dihydrolipoyl dehydrogenase (DD) (S31) which was up-regulated in, and is involved in the flavonoid and phenylpropanoid biosynthesis pathway. However, the exact role of DD in the defense mechanism is unclear (Tan et al., 2012).

Aldoketo reductases (AKR) (S15 and S16) are associated with various kinds of stress such as osmotic stress or dessication in barley (Bartels et al., 1991; Roncarati et al., 1995), oats (Li and Foley, 1995) and *Xerophyta viscosa* (Mundree et al., 2000) or protection against freezing in bromegrass (Lee and Chen, 1993) and in resistance mechanism linked to oxidative agents, salt, heavy metals and drought (Gavidia et al., 2002; Oberschall et al., 2000). In this study, spot S15 is down-regulated by 1.6 fold whereas Spot S16 is up-regulated by 1.4 folds at all the time points in resistant line 8-a. Both up- and down-regulation of AKR might be due to compromised resistance mechanism by switching off and on of various metabolic pathways such as phenylpropanoid pathways involved in plant defense. It has been shown that chalcone reductase

(CHR) involved in the flavonoid biosynthesis pathway originates from AKR (Naoumkina et al., 2010). It may also be involved in scavenging of ROS protecting the cells from the ROS toxicity (Li et al., 2011).

2.4.5 Rust affects nitrogen metabolism

Several ribosomal proteins (S19-S21), involved in protein synthesis (Carter et al., 2000), were up-regulated ranging from 1.1 to 1.6 folds during all the time points. It is possible that the protein biosynthesis is increased and is getting switched to energy generating pathways. This hypothesis is supported by the previous study (Tavernier et al., 2007) showing that upon infection, increased demand of energy results in the shuttling of amino acids into energy generating pathways such as the TCA cycle. For instance, glutamate dehydrogenase (GDH) can release amino nitrogen from amino acids to give keto-acid and NH_3 that can be recycled to be used in the TCA cycle and 20 protein amino acids can be metabolized into one of the seven intermediates (α-ketoglutarate, acetoacetate, acetyl-CoA, fumarate, oxaloacetate, pyruvate, and scuccinyl-coA) that are needed for energy generation in plants. In addition, it has been shown that nitrogen metabolism has a significant impact during the plant defense mechanism (Pageau et al., 2006; Stephenson et al., 1997). By shuttling nitrogen metabolism into energy generating pathways, plants can deprive pathogens of nutrients by actively mobilizing the nutrients away from the infection site (Newingham et al., 2007). Interestingly, nitrogen can also be directly involved in the defense mechanism through nitrogen species such as nitric oxide (NO). NO can prove toxic to the invading pathogen helping to ward off the pathogen along with the synergistic effect of ROS (Lamotte et al., 2004) triggering HR responses and other defense responses.

Gamma glutamyl hydrolase (S30 and S37) is the most abundant protein found in the soybean xylem sap (Krishnan et al., 2011). It is very interesting to know that xylem sap of soybean are rich in plant defense related proteins such as peroxidase, chitinase and serine protease (Krishnan et al., 2011). The xylem sap of several plants is shown to contain abundant defense proteins (Alvarez et al., 2006; Buhtz et al., 2004; Kehr et al., 2005). The up-regulation of these proteins in our study matches with these findings and indicates that the defense proteins may get induced in the xylem sap of soybean plants upon pathogen infection.

Spots S18 (XP_003537975), S27 (XP_003528797) and S29 (NP_001235654) were identified as proteins of unknown function.

To summarize the results of this study, out of 10 sibling lines, 5 sibling lines showed differential resistance responses against Louisiana rust isolates. A detached leaf assay and greenhouse screening showed similar responses using Louisiana rust isolates except the rust was generally more severe in the detached leaf assay. The results of the detached leaf and greenhouse assays showed low correlation mainly due to the lack of uniform environmental conditions required for disease development in the greenhouse. Based on the screening results lines 8-a, and 8-c were selected to compare protein expression in response to infection. Several differentially expressed proteins were observed between lines 8-a, and 8-c in response to rust inoculation and 37 proteins were identified using mass spectrometry. The different categories of proteins based upon their biological function fall into major groups like photosynthesis and metabolism, defense proteins, seed storage protein, protein metabolism etc. indicating that how the primary metabolism and secondary metabolism is being coordinated by altering the levels of different proteins at different time points to combat the pathogen attack. These results show that the differentially expressed

could be majorly involved in the resistance response to ASR infection. These results will be useful for further understanding of the biochemical pathways and molecular mechanisms of the host-pathogen interaction.

LITERATURE CITED

Abbink T.E., Peart J.R., Mos T.N., Baulcombe D.C., Bol J.F., Linthorst H.J. (2002) Silencing of a gene encoding a protein component of the oxygen-evolving complex of photosystem II enhances virus replication in plants. Virology 295:307-319.

Agarwal S., Saini R. (2009) Undescribed wheat gene for partial leaf rust and stripe rust resistance from Thatcher derivatives RL6058 and 90RN249 carryingLr34. Journal of Applied Genetics 50:199-204.

Akamatsu H., Yamanaka N., Yamaoka Y., Soares R.M., Morel W., Ivancovich A.J.G., Bogado A.N., Kato M., Yorinori J.T., Suenaga K. (2013) Pathogenic diversity of soybean rust in Argentina, Brazil, and Paraguay. Journal of General Plant Pathology 79:28-40. DOI: 10.1007/s10327-012-0421-7.

Alvarez S., Goodger J.Q., Marsh E.L., Chen S., Asirvatham V.S., Schachtman D.P. (2006) Characterization of the maize xylem sap proteome. Journal of Proteome Research 5:963-972.

Amme S., Matros A., Schlesier B., Mock H.P. (2006) Proteome analysis of cold stress response in Arabidopsis thaliana using DIGE-technology. Journal of Experimental Botany 57:1537-1546. DOI: 10.1093/jxb/erj129.

Asada K. (1999) The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annual Review of Plant Biology 50:601-639.

Bartels D., Engelhardt K., Roncarati R., Schneider K., Rotter M., Salamini F. (1991) An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related protein. The EMBO journal 10:1037.

Basha S.M., Mazhar H., Vasanthaiah H.K. (2010) Proteomics approach to identify unique xylem sap proteins in Pierce's disease-tolerant Vitis species. Applied Biochemistry and Biotechnology 160:932-944.

Beber R., de Francisco A., Alves A., De Sá R., Ogliari P. (2002) Chemical characterization of Brazilian oat genotypes. Acta científica venezolana 53:202.

Benedikz P., Mappledoram C.J., Scott P. (1981) A laboratory technique for screening cereals for resistance to *Septoria nodorum* using detached seedling leaves. Transactions of the British Mycological Society 77:667-669.

Berger S., Sinha A.K., Roitsch T. (2007) Plant physiology meets phytopathology: plant primary metabolism and plant-pathogen interactions. Journal of Experimental Botany 58:4019-4026. DOI: 10.1093/jxb/erm298.

Bernardo L., Prinsi B., Negri A.S., Cattivelli L., Espen L., Valè G. (2012) Proteomic characterization of the *Rph15* barley resistance gene-mediated defence responses to leaf rust. BMC Genomics 13:1-16.

Bolton M.D. (2009) Primary metabolism and plant defense-fuel for the fire. Molecular Plant-Microbe Interactions 22:487-497.

Bolton M.D., Kolmer J.A., Xu W.W., Garvin D.F. (2008) Lr34-mediated leaf rust resistance in wheat: transcript profiling reveals a high energetic demand supported by transient recruitment of multiple metabolic pathways. Molecular Plant-Microbe Interactions 21:1515-1527.

Bonde M.R., Nester S.E., Berner D.K., Frederick R.D. (2006) Effects of temperature on initiation of infection in soybean by isolates of *Phakopsora pachyrhizi* and *P meibomiae*. Phytopathology 96:S14-S14.

Bonfig K.B., Schreiber U., Gabler A., Roitsch T., Berger S. (2006) Infection with virulent and avirulent *P. syringae* strains differentially affects photosynthesis and sink metabolism in Arabidopsis leaves. Planta 225:1-12.

Bowler C., Van Camp W., Van Montagu M., Inzé D., Asada K. (1994) Superoxide dismutase in plants. Critical Reviews in Plant Sciences 13:199-218.

Bradford M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Analytical Biochemistry 72: 248-254.

Brechenmacher L., Lee J., Sachdev S., Song Z., Nguyen T.H.N., Joshi T., Oehrle N., Libault M., Mooney B., Xu D., Cooper B., Stacey G. (2009) Establishment of a protein reference map for soybean root hair cells. Plant Physiology 149:670-682. DOI: 10.1104/pp.108.131649.

Broman K.W. (2005) The genomes of recombinant inbred lines. Genetics 169:1133-1146.

Bromfield K.R. (1984) Soybean rust. Monograph, American Phytopathological Society.

Bromfield K.R., Hartwig E.E. (1980) Resistance to soybean rust and mode of inheritance. Crop Science 20:254-255.

Brown J., Wolfe M. (1990) Structure and evolution of a population of *Erysiphe graminis* f. sp. *hordei*. Plant Pathology 39:376-390.

Buhtz A., Kolasa A., Arlt K., Walz C., Kehr J. (2004) Xylem sap protein composition is conserved among different plant species. Planta 219:610-618.

Burdon J.J. (1988) Major gene resistance to *Phakopsora pachyrhizi* in *Glycine canscens*, a wild relative of soybean. Theoretical and Applied Genetics 75:923-928.

Burdon J.J., Marshall D.R. (1981) Interspecific and intraspecific diversity in the disease-response of Glycine species to the leaf rust fungus *Phakopsora pachyrhizi*. Journal of Ecology 69:381-390.

Caldwell C.R., Turano F.J., McMahon M.B. (1997) Identification of two cytosolic ascorbate peroxidase cDNAs from soybean leaves and characterization of their products by functional expression in *E. coli*. Planta 204:120-126.

Candiano G., Bruschi M., Musante L., Santucci L., Ghiggeri G.M., Carnemolla B., Orecchia P., Zardi L., Righetti P.G. (2004) Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25:1327-1333.

Carter A.P., Clemons W.M., Brodersen D.E., Morgan-Warren R.J., Wimberly B.T., Ramakrishnan V. (2000) Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature 407:340-348.

Castillejo M.A., Maldonado A.M., Dumas-Gaudot E., Fernandez-Aparicio M., Susin R., Diego R., Jorrin J.V. (2009) Differential expression proteomics to investigate responses and resistance to *Orobanche crenata* in *Medicago truncatula*. BMC Genomics 10. DOI: 10.1186/1471-2164-10-294.

Chatfield M., Dalton D.A. (1993) Ascorbate peroxidase from soybean root nodules. Plant Physiology 103:661.

Chen Z., Brown R., Guo B., Menkir A., Cleveland T. (2009) Identifying Aflatoxin Resistance-related Proteins/Genes through Proteomics and RNAi Gene Silencing 1. Peanut Science 36:35-41.

Chen Z.Y., Brown R.L., Cleveland T.E. (2004) Evidence for an association in corn between stress tolerance and resistance to *Aspergillus flavus* infection and aflatoxin contamination. African Journal of Biotechnology 3:693-699.

Cheng Y., Chan K. (1968) The breeding of rust resistant soybean Tainung 3. Journal of Taiwan Agricultural Research 17:30-34.

Choi J.J., Alkharouf N.W., Schneider K.T., Matthews B.F., Frederick R.D. (2008) Expression patterns in soybean resistant to *Phakopsora pachyrhizi* reveal the importance of peroxidases and lipoxygenases. Functional & Integrative Genomics 8:341-359. DOI: 10.1007/s10142-008-0080-0.

Chou H.M., Bundock N., Rolfe S.A., Scholes J.D. (2000) Infection of *Arabidopsis thaliana* leaves with *Albugo candida* (white blister rust) causes a reprogramming of host metabolism. Molecular Plant Pathology 1:99-113.

Chung G., Singh R.J. (2008) Broadening the genetic base of soybean: A multidisciplinary approach. Critical Reviews in Plant Sciences 27:295-341. DOI: 10.1080/07352680802333904.

Cooper B., Campbell K.B., Feng J., Garrett W.M., Frederick R. (2011) Nuclear proteomic changes linked to soybean rust resistance. Molecular Biosystems 7:773-783.

Costa P., Plomion C. (1999) Genetic analysis of needle proteins in maritime pine. 2. Variation of protein accumulation. Silvae Genetica 48:146-150.

Crowell D.N., John M.E., Russell D., Amasino R.M. (1992) Characterization of a stress-induced, developmentally regulated gene family from soybean. Plant Molecular Biology 18:459-466.

Dalton D.A., Russell S.A., Hanus F., Pascoe G.A., Evans H.J. (1986) Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. Proceedings of the National Academy of Sciences 83:3811-3815.

Dalton D.A., del Castillo L.D., Kahn M.L., Joyner S.L., Chatfield J.M. (1996) Heterologous expression and characterization of soybean cytosolic ascorbate peroxidase. Archives of Biochemistry and Biophysics 328:1-8.

Daniel S.L., Hartman G.L., Wagner E.D., Plewa M.J. (2007) Mammalian cell cytotoxicity analysis of soybean rust fungicides. Bulletin of Environmental Contamination and Toxicology 78:474-478. DOI: 10.1007/s00128-007-9193-8.

De Mortel M.V., Schneider K.T., Bancroft T., Nettleton D., Frederick R.D., Baum T.J., Whitham S.A. (2007) Gene expression in a soybean cultivar containing the *Rpp3* gene for resistance to *Phakopsora pachyrhizi*. Phytopathology 97:S117.

Diamond H., Cooke B. (1999) Towards the development of a novel in vitro strategy for early screening of Fusarium ear blight resistance in adult winter wheat plants. European Journal of Plant Pathology 105:363-372.

Doehlemann G., van der Linde K., Aßmann D., Schwammbach D., Hof A., Mohanty A., Jackson D., Kahmann R. (2009) Pep1, a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. Plos Pathogens 5:e1000290.

Dornez E., Croes E., Gebruers K., Carpentier S., Swennen R., Laukens K., Witters E., Urban M., Delcour J.A., Courtin C.M. (2010) 2-D DIGE reveals changes in wheat xylanase inhibitor protein families due to *Fusarium graminearum ΔTri5* infection and grain development. Proteomics 10:2303-2319. DOI: 10.1002/pmic.200900493.

Dorrance A.E., Draper M.A., Hershman D.E. (2007) Using foliar fungicides to manage soybean rust. DOI: http://oardc.osu.edu/soyrust/2007edition/fungisoyrust.pdf.

Ezawa S., Tada Y. (2009) Identification of salt tolerance genes from the mangrove plant *Bruguiera gymnorhiza* using Agrobacterium functional screening. Plant Science 176:272-278.

Feder M.E., Hofmann G.E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annual review of physiology 61:243-282.

Fernandez-Ortuño D., Tores J.A., de Vicente A., Perez-Garcia A. (2008) First report of powdery mildew elicited by *Podosphaera fusca* (Synonym *Podosphaera xanthii*) on *Euryops pectinatus* in Spain. Plant Disease 92:835-835. DOI: 10.1094/pdis-92-5-0835c.

Freire M.C.M., de Oliveira L.O., de Almeida A.M.R., Schuster I., Moreira M.A., Liebenberg M.M., Mienie C.M.S. (2008) Evolutionary history of *Phakopsora pachyrhizi* (the Asian soybean rust) in Brazil based on nucleotide sequences of the internal transcribed spacer region of the nuclear ribosomal DNA. Genetics and Molecular Biology 31:920-931.

Garcia A., Calvo E.S., Kiihl R.A.D., Harada A., Hiromoto D.M., Vieira L.G.E. (2008) Molecular mapping of soybean rust (*Phakopsora pachyrhizi*) resistance genes: discovery of a novel locus and alleles. Theoretical and Applied Genetics 117:545-553. DOI: 10.1007/s00122-008-0798-z.

Gavidia I., Pérez-Bermúdez P., Seitz H.U. (2002) Cloning and expression of two novel aldo-keto reductases from *Digitalis purpurea* leaves. European Journal of Biochemistry 269:2842-2850.

Gorg A., Obermaier C., Boguth G., Weiss W. (1999) Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: Wide pH gradients up to pH 12, longer separation distances and simplified procedures. Electrophoresis 20:712-717.

Gorg A., Obermaier C., Boguth G., Harder A., Scheibe B., Wildgruber R., Weiss W. (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21:1037-1053.

Gygi S.P., Rochon Y., Franza B.R., Aebersold R. (1999) Correlation between protein and mRNA abundance in yeast. Molecular and Cellular Biology 19:1720-1730.

Hajduch M., Tanaka H., Morinaka Y., Otake Y., Nakamura H., Kayano T., Koga-Ban Y. (2005) Protein analysis of dwarfed transgenic rice plants overexpressing GA2-oxidase gene. Biologia Plantarum 49:621-624. DOI: 10.1007/s10535-005-0061-2.

Hartman G.L., Wang T.C., Hymowitz T. (1992) Sources of resistance to soybean rust in perennial Glycine species. Plant Disease 76:396-399.

Hartman G.L., Miles M.R., Frederick R.D. (2005) Breeding for resistance to soybean rust. Plant Disease 89:664-666. DOI: 10.1094/pd-89-0664.

Hartwig E., Bromfield K.R. (1983) Relationships among three genes conferring specific resistance to rust in soybeans. Crop Science 23:237-239.

Hartwig E.E. (1986) Identification of a 4th major gene conferring resistance to soybean rust. Crop Science 26:1135-1136.

Hempel J., Zehner S., Göttfert M., Patschkowski T. (2009) Analysis of the secretome of the soybean symbiont *Bradyrhizobium japonicum*. Journal of Biotechnology 140:51-58.

Hidayat O.O., Somaatmadja S. (1977) Screening of soybean breeding lines for resistance to soybean rust (*Phakopsora pachyrhizi* Sydow). Soybean Rust Newsletter 1:9-22.

Hurkman W.J., Tanaka C.K. (1986) Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiology 81:802-806.

Hymowitz T. (1980) Chemical germplasm investigations in soybeans: the flotsam hypothesis. Swain, T.; Kleiman, R. The resource potential in phytochemistry:157-179.

Hymowitz T. (1995) Soybean: Glycine max (Leguminosae: Papilionoidae). Evolution of Crop Plants. J. Smarzz and NW Simmonds, eds. Longman Scientific & Technical, Harlow:261-266.

Jakobek J., Lindgren P. (2002) Expression of a bean acid phosphatase cDNA is correlated with disease resistance. Journal of Experimental Botany 53:387-389.

Jansen R.C., Nap J.P., Mlynárová L. (2002) Errors in genomics and proteomics. Nature Biotechnology 20:19.

Jarosz A.M., Burdon J.J. (1990) Predominance of a single major gene for resistance to *Phakopsora pachyrhizi* in a population of *Glycine argyrea*. Heredity 64:347-353.

Joseph L.M., Hymowitz T., Schmidt M.A., Herman E.M. (2006) Evaluation of Glycine Germplasm for Nulls of the Immunodominant Allergen P34/Gly m Bd 30k. Crop Science 46:1755–1763.

Jurick W.N., Narvaez D.F., Brennan M.M., Harmon C.L., Marois J.J., Wright D.L., Harmon P.F. (2008) Winter survival of the soybean rust pathogen, *Phakopsora pachyrhizi*, in Florida. Plant Disease 92:1551-1558. DOI: 10.1094/pdis-92-11-1551.

Kaneda T., Fujiwara S., Takai R., Takayama S., Isogai A., Che F.-S. (2007) Identification of genes involved in induction of plant hypersensitive cell death. Plant Biotechnology 24:191-200.

Kanzaki H., Saitoh H., Ito A., Fujisawa S., Kamoun S., Katou S., Yoshioka H., Terauchi R. (2003) Cytosolic HSP90 and HSP70 are essential components of INF1-mediated hypersensitive response and non-host resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana*. Molecular Plant Pathology 4:383-391. DOI: 10.1046/j.1364-3703.2003.00186.x.

Kehr J., Buhtz A., Giavalisco P. (2005) Analysis of xylem sap proteins from *Brassica napus*. BMC Plant Biology 5:11.

Killgore E., Heu R., Gardner D.E. (1994) First report of soybean rust in Hawaii. Plant Disease 78:1216-1216.

Kim K.S., Wang T.C., Yang X.B. (2005) Simulation of apparent infection rate to predict severity of soybean rust using a fuzzy logic system. Phytopathology 95:1122-1131. DOI: 10.1094/phyto-95-1122.

Kim S.T., Kim S.G., Hwang D.H., Kang S.Y., Kim H.J., Lee B.H., Lee J.J., Kang K.Y. (2004) Proteomic analysis of pathogen responsive proteins from rice leaves induced by rice blast fungus, *Magnaporthe grisea*. Proteomics 4:3569-3578.

Kleine-Tebbe J., Wangorsch A., Vogel L., Crowell D.N., Haustein U.-F., Vieths S. (2002) Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1–related PR-10 protein in soybean, SAM22. Journal of Allergy and Clinical Immunology 110:797-804.

Kramer C.Y. (1956) Extension of multiple range tests to group means with unequal numbers of replications. Biometrics 12:307-310.

Krishnan H.B., Natarajan S.S., Bennett J.O., Sicher R.C. (2011) Protein and metabolite composition of xylem sap from field-grown soybeans (Glycine max). Planta 233:921-931.

Kumudini S., Godoy C.V., Board J.E., Omielan J., Tollenaar M. (2008) Mechanisms involved in soybean rust-induced yield reduction. Crop Science 48:2334-2342. DOI: 10.2135/cropsci2008.01.0009.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lamotte O., Gould K., Lecourieux D., Sequeira-Legrand A., Lebrun-Garcia A., Durner J., Pugin A., Wendehenne D. (2004) Analysis of nitric oxide signaling functions in tobacco cells challenged by the elicitor cryptogein. Plant Physiology 135:516-529.

Lee S.P., Chen T.H. (1993) Molecular cloning of abscisic acid-responsive mRNAs expressed during the induction of freezing tolerance in bromegrass (*Bromus inermis* Leyss) suspension culture. Plant Physiology 101:1089-1096.

Levy C. (2005) Epidemiology and chemical control of soybean rust in Southern Africa. Plant Disease 89:669-674. DOI: 10.1094/pd-89-0669.

Li B., Foley M.E. (1995) Cloning and characterization of differentially expressed genes in imbibed dormant and afterripened *Avena fatua* embryos. Plant Molecular Biology 29:823-831.

Li K., Xu C., Zhang J. (2011) Proteome profile of maize (*Zea Mays* L.) leaf tissue at the flowering stage after long-term adjustment to *rice black-streaked dwarf virus* infection. Gene 485:106-113.

Li S.X., Smith J.R., Ray J.D., Frederick R.D. (2012) Identification of a new soybean rust resistance gene in PI 567102B. Theoretical and Applied Genetics 125:133-142. DOI: 10.1007/s00122-012-1821-y.

Liu D., Xia X.C., He Z.H., Xu S.C. (2008) A novel homeobox-like gene associated with reaction to stripe rust and powdery mildew in common wheat. Phytopathology 98:1291-1296.

Liu Y., Ahn J.-E., Datta S., Salzman R.A., Moon J., Huyghues-Despointes B., Pittendrigh B., Murdock L.L., Koiwa H., Zhu-Salzman K. (2005) Arabidopsis vegetative storage protein is an anti-insect acid phosphatase. Plant Physiology 139:1545-1556.

Lodha T.D., Hembram P., Tep N., Basak J. (2013) Proteomics: A Successful Approach to Understand the Molecular Mechanism of Plant-Pathogen Interaction. American Journal of Plant Sciences 4:1212-1226.

Lu G., Jantasuriyarat C., Zhou B., Wang G.-L. (2004) Isolation and characterization of novel defense response genes involved in compatible and incompatible interactions between rice and *Magnaporthe grisea*. Theoretical and Applied Genetics 108:525-534.

McLean R.J., Byth D.E. (1980a) Inheritance of resistance to rust (*Phakopsora pachyrhizi*) in soybeans. Australian Journal of Agricultural Research 31:951-956.

McLean R.J., Byth D.E. (1980b) Inheritance of resistance to rust (*Phakopsora pachyrhizi*) in soybeans. Australian Journal of Agricultural Research 31:951-956.

McLean R.J., Byth D.E. (1981) Histological studies of the pre-penetration development and penetration of soybeans by rust, *Phakopsora pachyrhizi* Syd. Australian Journal of Agricultural Research 32:435-443.

Menkir A., Brown R.L., Bandyopadhyay R., Chen Z.Y., Cleveland T.E. (2006) A USA-Africa collaborative strategy for identifying, characterizing, and developing maize germplasm with resistance to aflatoxin contamination. Mycopathologia 162:225-232. DOI: 10.1007/s11046-006-0056-3.

Merril C. (1990) Silver staining of proteins and DNA. Nature 343:779-780.

Miles M., Morel W., Steinlage T., Hartman G. (2003a) Summary of the USDA fungicide efficacy trials to control soybean rust in Paraguay 2003–2004. Online. USDA National Information System for the Regional IPM Centers. USDA, Washington, DC.

Miles M.R., Hartman G.L., Levy C., Morel W. (2003b) Current status of soybean rust control by fungicides. Pesticide Outlook 14:197-200.

Miles M.R., Levy C., Morel W., Mueller T., Steinlage T., van Rij N., Frederick R.D., Hartman G.L. (2007) International fungicide efficacy trials for the management of soybean rust. Plant Disease 91:1450-1458. DOI: 10.1094/pdis-91-11-1450.

Mittler R. (2002) Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science 7:405-410.

Moreno J.I., Martín R., Castresana C. (2005) Arabidopsis SHMT1, a serine hydroxymethyltransferase that functions in the photorespiratory pathway influences resistance to biotic and abiotic stress. The Plant Journal 41:451-463.

Mueller T.A., Miles M.R., Morel W., Marois J.J., Wright D.L., Kemerait R.C., Levy C., Hartman G.L. (2009) Effect of Fungicide and Timing of Application on Soybean Rust Severity and Yield. Plant Disease 93:243-248. DOI: 10.1094/pdis-93-3-0243.

Mundree S.G., Whittaker A., Thomson J.A., Farrant J.M. (2000) An aldose reductase homolog from the resurrection plant *Xerophyta viscosa* Baker. Planta 211:693-700.

Musrati R., Kollarova M., Mernik N., Mikulasova D. (1998) Malate dehydrogenase: distribution, function and properties. General physiology and biophysics 17:193-210.

Naoumkina M.A., Zhao Q., Gallego-Giraldo L., Dai X., Zhao P.X., Dixon R.A. (2010) Genome-wide analysis of phenylpropanoid defence pathways. Molecular Plant Pathology 11:829-846.

Newingham B.A., Callaway R.M., BassiriRad H. (2007) Allocating nitrogen away from a herbivore: a novel compensatory response to root herbivory. Oecologia 153:913-920.

Nunes-Nesi A., Carrari F., Lytovchenko A., Smith A.M., Loureiro M.E., Ratcliffe R.G., Sweetlove L.J., Fernie A.R. (2005) Enhanced photosynthetic performance and growth as a consequence of decreasing mitochondrial malate dehydrogenase activity in transgenic tomato plants. Plant Physiology 137:611-622.

Oberschall A., Deák M., Török K., Sass L., Vass I., Kovács I., Fehér A., Dudits D., Horváth G.V. (2000) A novel aldose/aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stresses. The Plant Journal 24:437-446.

Ochoa-Acuña H.G., Bialkowski W., Yale G., Hahn L. (2009) Toxicity of soybean rust fungicides to freshwater algae and *Daphnia magna*. Ecotoxicology 18:440-446. DOI: 10.1007/s10646-009-0298-1.

Ogle H.J., Byth D.E., McLean R. (1979) Effect of rust (*Phakopsora pachyrhizi*) on soybean yield and quality in Southeastern Queensland. Australian Journal of Agricultural Research 30:883-893.

Okinaka Y., Yang C.-H., Herman E., Kinney A., Keen N.T. (2002) The P34 syringolide elicitor receptor interacts with a soybean photorespiration enzyme, NADH-dependent hydroxypyruvate reductase. Molecular Plant-Microbe Interactions 15:1213-1218.

Ono Y., Buritica P., Hennen J.F. (1992) Delimitation of *Phakopsora*, *Physopella* and *Cerotelium* and their species on leguminosae. Mycological Research 96:825-850.

Pageau K., Reisdorf-Cren M., Morot-Gaudry J.F., Masclaux-Daubresse C. (2006) The two senescence-related markers, *GS1* (cytosolic glutamine synthetase) and *GDH* (glutamate dehydrogenase), involved in nitrogen mobilization, are differentially regulated during pathogen attack and by stress hormones and reactive oxygen species in *Nicotiana tabacum* L. leaves. Journal of Experimental Botany 57:547-557. DOI: 10.1093/jxb/erj035.

Pan Z., Yang X.B., Pivonia S., Xue L., Pasken R., Roads J. (2006) Long-term prediction of soybean rust entry into the Continental United States. Plant Disease 90:840-846. DOI: 10.1084/pd-90-0840.

Panthee D.R., Yuan J.S., Wright D.L., Marois J.J., Mailhot D., Stewart C.N. (2007) Gene expression analysis in soybean in response to the causal agent of Asian soybean rust (*Phakopsora pachyrhizi* Sydow) in an early growth stage. Functional & Integrative Genomics 7:291-301. DOI: 10.1007/s10142-007-0045-8.

Park S., Chen Z., Ganiger M.C., Fortunato A.A. (2010) Protein profile differences between soybean accessions resistant and susceptible to soybean rust (*Phakopsora pachyrhizi*). Phytopathology 100:S96-S96.

Park S., Chen Z.Y., Chanda A.K., Schneider R.W., Hollier C.A. (2008) Viability of *Phakopsora pachyrhizi* urediniospores under simulated southern Louisiana winter temperature conditions. Plant Disease 92:1456-1462. DOI: 10.1094/pdis-92-10-1456.

Patil P., Anahosur K. (1998) Control of soybean rust by fungicides. Indian Phytopathology 51:265-268.

Patton W.F. (2000) Making blind robots see: the synergy between fluorescent dyes and imaging devices in automated proteomics. Biotechniques 28:944.

Patzoldt M.E., Tyagi R.K., Hymowitz T., Miles M.R., Hartman G.L., Frederick R.D. (2007) Soybean rust resistance derived from *Glycine tomentella* in amphiploid hybrid lines. Crop Science 47:158-161. DOI: 10.2135/cropsci2006.05.0328.

Paul C., Hartman G.L. (2009) Sources of Soybean Rust Resistance Challenged with Single-Spored Isolates of *Phakopsora pachyrhizi*. Crop Science 49:1781-1785. DOI: 10.2135/cropsci2008.12.0710.

Pérez-de-Luque A., Lozano M., Maldonado A., Jorrín J., Dita J., Román B., Rubiales D. (2007) *Medicago truncatula* as a model for studying interactions between root parasitic plants

and legumes, in: U. Mathesius, et al. (Eds.), The Medicago truncatula handbook, The Samuel Roberts Noble Foundation, Ardmore. pp. 1-31.

Perkins D.N., Pappinl D.J.C., Creasy D.M., Cottrell J.S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20:3551-3567.

Pham T.A., Miles M.R., Frederick R.D., Hill C.B., Hartman G.L. (2009) Differential responses of resistant soybean entries to isolates of *Phakopsora pachyrhizi*. Plant Disease 93:224-228.

Pham T.A., Hill C.B., Miles M.R., Nguyen B.T., Vu T.T., Vuong T.D., VanToai T.T., Nguyen H.T., Hartman G.L. (2010) Evaluation of soybean for resistance to soybean rust in Vietnam. Field Crops Research 117:131-138. DOI: 10.1016/j.fcr.2010.02.011.

Pivonia S., Yang X.B. (2004) Assessment of the potential year-round establishment of soybean rust throughout the world. Plant Disease 88:523-529.

Pivonia S., Yang X.B. (2005) Assessment of epidemic potential of soybean rust in the United States. Plant Disease 89:678-682. DOI: 10.1094/pd-89-0678.

Pivonia S., Yang X.B. (2006) Relating epidemic progress from a general disease model to seasonal appearance time of rusts in the united states: Implications for soybean rust. Phytopathology 96:400-407. DOI: 10.1094/phyto-96-0400.

Rabilloud T. (2000) Proteome research: two-dimensional gel electrophoresis and identification methods Springer verlag.

Rampitsch C., Srinivasan M. (2006) The application of proteomics to plant biology: a review. Canadian Journal of Botany 84:883-892. DOI: 10.1139/b06-061.

Roncarati R., Salamini F., Bartels D. (1995) An aldose reductase homologous gene from barley: regulation and function. The Plant Journal 7:809-822.

Rupe J., Sconyers L. (2008) Soybean rust. The Plant Health Instructor. DOI: 10.1094/PHI-I-2008-0401-01.

Schneider R.W., Hollier C.A., Whitam H.K., Palm M.E., McKemy J.M., Hernandez J.R., Levy L., DeVries-Paterson R. (2005) First report of soybean rust caused by *Phakopsora pachyrhizi* in the continental United States. Plant Disease 89:774-774. DOI: 10.1094/pd-89-0774a.

Schoen D.J., Burdon J.J., Brown A.H.D. (1992) Resistance of *Glycine tomentella* to soybean leaf rust *Phakopsora pachyrhizi* in relation to ploidy level and geographic distribution. Theoretical and Applied Genetics 83:827-832.

Shevchenko A., Tomas H., Havlis J., Olsen J.V., Mann M. (2007) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nature Protocols 1:2856-2860.

Shi F., Yamamoto R., Shimamura S., Hiraga S., Nakayama N., Nakamura T., Yukawa K., Hachinohe M., Matsumoto H., Komatsu S. (2008) Cytosolic ascorbate peroxidase 2 (cAPX 2) is involved in the soybean response to flooding. Phytochemistry 69:1295-1303.

Singh B., Thapliyal P. (1977) Breeding for resistance to soybean rust in India. pp. 62-65.

Sørhagen K., Laxa M., Peterhänsel C., Reumann S. (2013) The emerging role of photorespiration and non-photorespiratory peroxisomal metabolism in pathogen defence. Plant Biology.

Soria-Guerra R.E., Rosales-Mendoza S., Chang S.Y., Haudenshield J.S., Padmanaban A., Rodriguez-Zas S., Hartman G.L., Ghabrial S.A., Korban S.S. (2010) Transcriptome analysis of resistant and susceptible genotypes of *Glycine tomentella* during *Phakopsora pachyrhizi* infection reveals novel rust resistance genes. Theoretical and Applied Genetics 120:1315-1333. DOI: 10.1007/s00122-009-1258-0.

Steinberg T.H., Haugland R.P., Singer V.L. (1996) Applications of SYPRO Orange and SYPRO Red protein gel stains. Analytical Biochemistry 239:238-245.

Stephenson S.A., Green J.R., Manners J.M., Maclean D.J. (1997) Cloning and characterisation of *glutamine synthetase* from *Colletotrichum gloeosporioides* and demonstration of elevated expression during pathogenesis on *Stylosanthes guianensis*. Current Genetics 31:447-454. DOI: 10.1007/s002940050228.

Szebenyi D.M., Musayev F.N., di Salvo M.L., Safo M.K., Schirch V. (2004) Serine hydroxymethyltransferase: role of glu75 and evidence that serine is cleaved by a retroaldol mechanism. Biochemistry 43:6865-6876.

Taler D., Galperin M., Benjamin I., Cohen Y., Kenigsbuch D. (2004) Plant eR genes that encode photorespiratory enzymes confer resistance against disease. The Plant Cell Online 16:172-184.

Tan E.C., Karsani S.A., Foo G.T., Wong S.M., Rahman N.A., Khalid N., Othman S., Yusof R. (2012) Proteomic analysis of cell suspension cultures of *Boesenbergia rotunda* induced by phenylalanine: identification of proteins involved in flavonoid and phenylpropanoid biosynthesis pathways. Plant Cell, Tissue and Organ Culture (PCTOC) 111:219-229.

Tavernier V., Cadiou S., Pageau K., Laugé R., Reisdorf-Cren M., Langin T., Masclaux-Daubresse C. (2007) The plant nitrogen mobilization promoted by *Colletotrichum lindemuthianum* in *Phaseolus* leaves depends on fungus pathogenicity. Journal of Experimental Botany 58:3351-3360.

Tremblay A., Hosseini P., Alkharouf N.W., Li S.X., Matthews B.F. (2010) Transcriptome analysis of a compatible response by *Glycine max* to *Phakopsora pachyrhizi* infection. Plant Science 179:183-193. DOI: 10.1016/j.plantsci.2010.04.011.

Twizeyimana M., Ojiambo P., Hartman G., Bandyopadhyay R. (2011) Dynamics of Soybean Rust Epidemics in Sequential Plantings of Soybean Cultivars in Nigeria. Plant Disease 95:43-50.

Twizeyimana M., Ojiambo P., Ikotun T., Paul C., Hartman G., Bandyopadhyay R. (2007) Comparison of field, greenhouse, and detached-leaf evaluations of soybean germplasm for resistance to *Phakopsora pachyrhizi*. Plant Disease 91:1161-1169.

Ünlü M., Morgan M.E., Minden J.S. (1997) Difference gel electrophoresis. A single gel method for detecting changes in protein extracts. Electrophoresis 18:2071-2077.

van de Mortel M., Recknor J.C., Graham M.A., Nettleton D., Dittman J.D., Nelson R.T., Godoy C.V., Abdelnoor R.V., Almeida A.M.R., Baum T.J., Whitham S.A. (2007) Distinct biphasic mRNA changes in response to Asian soybean rust infection. Molecular Plant-Microbe Interactions 20:887-899. DOI: 10.1094/mpmi-20-8-0887.

Vleeshouwers V.G., van Dooijeweert W., Keizer L.P., Sijpkes L., Govers F., Colon L.T. (1999) A laboratory assay for *Phytophthora infestans* resistance in various Solanum species reflects the field situation. European Journal of Plant Pathology 105:241-250.

Walker D.R., Boerma H.R., Phillips D.V., Schneider R.W., Buckley J.B., Shipe E.R., Mueller J.D., Weaver D.B., Sikora E.J., Moore S.H., Hartman G.L., Miles M.R., Harris D.K., Wright D.L., Marois J.J., Nelson R.L. (2011) Evaluation of USDA Soybean Germplasm Accessions for Resistance to Soybean Rust in the Southern United States. Crop Science 51:678-693. DOI: 10.2135/cropsci2010.06.0340.

Wan J.R., Torres M., Ganapathy A., Thelen J., DaGue B.B., Mooney B., Xu D., Stacey G. (2005) Proteomic analysis of soybean root hairs after infection by *Bradyrhizobium japonicum*. Molecular Plant-Microbe Interactions 18:458-467. DOI: 10.1094/mpmi-18-0458.

Wang Y., Yuan X.Z., Hu H., Liu Y., Sun W.H., Shan Z.H., Zhou X.A. (2012) Proteomic analysis of differentially expressed proteins in resistant soybean leaves after *Phakopsora pachyrhizi* infection. Journal of Phytopathology 160:554-560. DOI: 10.1111/j.1439-0434.2012.01949.x.

Watson B.S., Asirvatham V.S., Wang L., Sumner L.W. (2003) Mapping the proteome of barrel medic (*Medicago truncatula*). Plant Physiology 131:1104.

Wildgruber R., Harder A., Obermaier C., Boguth G., Weiss W., Fey S.J., Larsen P.M., Görg A. (2000) Towards higher resolution: Two dimensional Electrophoresis of *Saccharomyces cerevisiae* proteins using overlapping narrow immobilized pH gradients. Electrophoresis 21:2610-2616.

Xu C., Garrett W.M., Sullivan J., Caperna T.J., Natarajan S. (2006) Separation and identification of soybean leaf proteins by two-dimensional gel electrophoresis and mass spectrometry. Phytochemistry 67:2431-2440.

Xu C., Sullivan J.H., Garrett W.M., Caperna T.J., Natarajan S. (2008) Impact of solar ultraviolet-B on the proteome in soybean lines differing in flavonoid contents. Phytochemistry 69:38-48.

Yang X.B., Dowler W.M., Tschanz A.T. (1991) A Simulation Model For Assessing Soybean rust epidemics. Journal of Phytopathology 133:187-200.

Yang X.B., Royer M.H., Tschanz A.T., Tsai B.Y. (1990) Analysis and quantification of soybean rust epidemics from 73 sequential planting experiments. Phytopathology 80:1421-1427.

Yorinori J.T., Paiva W.M., Frederick R.D., Costamilan L.M., Bertagnolli P.F., Hartman G.E., Godoy C.V., Nunes J. (2005) Epidemics of soybean rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003. Plant Disease 89:675-677. DOI: 10.1094/pd-89-0675.

Zou J., Singh R., Hymowitz T. (2004) SSR marker and ITS cleaved amplified polymorphic sequence analysis of soybean× *Glycine tomentella* intersubgeneric derived lines. Theoretical and Applied Genetics 109:769-774.

VITA

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