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Soybean Rust: Five Years of Research

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1. Introduction

Soybean rust (SR), *Phakopsora pachyrhizi* Sydow is an obligate biotrophic fungal pathogen causing rust on a wide range of hosts including many kind of beans (*Glycine max*, *Phaseolus vulgaris* var *vulgaris*, *P. lunatus* var *lunatus*, *P. coccineus*, *Vicia faba*, *Lablab purpureus*, *Vigna radiata*, *V. unguiculata*, *Psophocarpus tetragonogobus*, *Pachyrhizus ahipa*, *P. erosus*), few kind of peas (*Pisum sativum*, *Cajanus cajan*), many kinds of clover (*Alisycarpus vaginalis*, *Trifolium repens*, *T. incarnatum*, *Melilotus officinalis*), fenugreek, lupines, trefoil and kudzu. It originated in Asia and emerged in the continental United States (Louisiana) in 2004 (Scheinder et al., 2005) following hurricane Ivan which brought SR spores from Columbia. Presence of SR in South America since 2001 has been responsible for large yield losses in soybean, *Glycine max* L. Merr., fields. For example, in Brazil, a recent study depicted a two-year field trial where soybean rust was responsible for 37% to 67% of soybean seed yield losses (Kumudini et al., 2008). This study agreed with yield losses already observed in Asia, where the disease originated. Losses there can reach up to 80% (Miles et al., 2003). Based on a disease risk assessment study conducted by Pivonia and Yang (2005), climatic conditions in the soybean producing regions of the United States are suitable for similar yield losses. In 2009, soybean rust has been detected in sixteen states including 576 counties along the southeastern portion of the country (USDA Integrated Pest Management (IPM) web site; http://sbr.ipmpipe.org/cgi-bin/sbr/public.cgi). Its location boundaries in the US are mostly due to its warm location preference in addition to the presence of an alternative host, kudzu, in which it can overwintered. The invasive kudzu plant is an alternative hosts and its massive presence in several locations in South Louisiana and along the Mississippi river makes these locations of choice for soybean rust. Numerous studies have been conducted to build a system model allowing prediction, detection and evaluation of the presence of SR in soybean fields (Tao et al., 2009; Roberts et al., 2009).

1.1 Phakopsora pachyrhizi life cycle

The lifecycle of *P. pachyrhizi* is typical of the majority of other rust fungi with some particularities. Spores, named uredospores, are transported readily by air currents and can be disseminated hundreds of miles in a few days. Weather conditions determine when the spores begin their infection cycle, since they need a period where the temperature fluctuates between 15°C and 28°C with high humidity for germination (Melching et al., 1989). When germination occurs, the uredospore produces a single germ tube that grows across the leaf

surface until an appressorium forms. Appressoria form over anticlinal walls or over the center of epidermal cells, but rarely over stomata, in contrast to the habit of many other rusts. Thus, penetration is direct rather than through natural openings or through wounds in the leaf tissue. Penetration hyphae, stemming from the appressorium cone, pass through the cuticle to emerge in the intercellular space where a septum is formed to produce the primary hyphae. The primary hypha grows between spongy mesophyll cells where it forms the haustorium. This new infection structure is a specialized organ produced to allow fungi to obtain nutrients from the host. Other functions such as suppression of host defense responses, redirection or reprogramming of the host's metabolic flow, and biosynthesis might be associated with this specialized organ, although direct evidence to date is lacking (Voegele et al., 2004).

Once this first stage has been reached, additional hyphae emerge and spread through the apoplast where many other haustoria are formed. At approximately 5 days after infection, some necrosis of epidermal and mesophyll cells occurs that is visible at the upper surface of the leaves as yellow mosaic discolorations. These symptoms appeared the same on a resistant and a susceptible cultivar (Figure 1A). Hyphae aggregate and a uredinium arises in
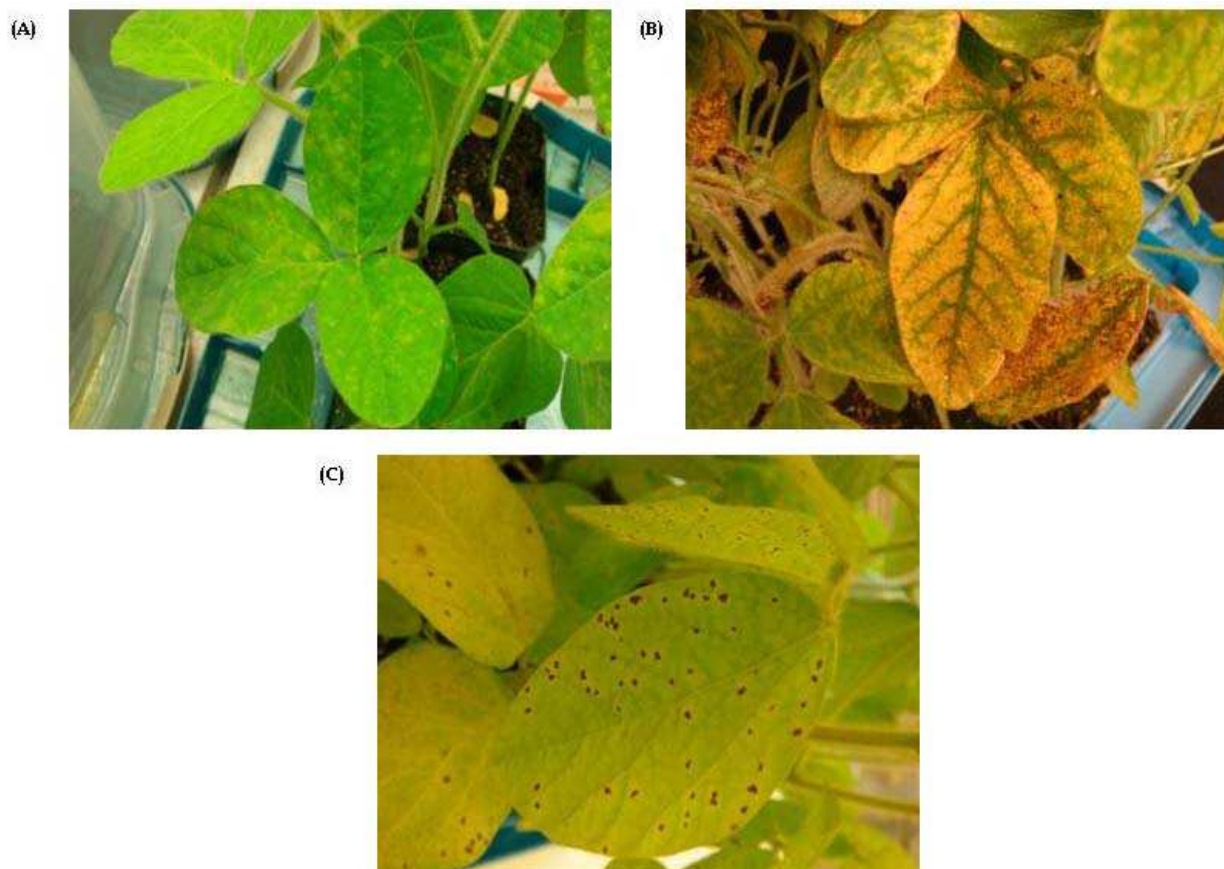

Fig. 1. SR symptoms observed on soybean leaves. (a) Yellow mosaic discoloration observed at 7 dai. (b) Tan lesions observed at 21 dai and (c) reddish-brown lesions observed at 14 dai. Photos were taken at the United States Department of Agriculture-Agricultural Research Service Stoneville Research Quarantine Facility in Mississippi.

the spongy mesophyll cell layer. Uredinia can develop 5 to 8 days after infection by a uredospore and its development might extend up to 4 weeks. The first uredospores produced by the uredinia arise 9 to 10 days after initial infection. Figure 2 shows SR development on a leaf. Spore production can be observed for up to 3 weeks. Secondary uredinia may arise on the margins of the initial infection and extend spore production for an additional 8 weeks. Thus, from an initial infection, there can be first generation uredinia that maintain sporulation for up to 15 weeks (Koch et al., 1983; Mendgen and Hahn, 2002). This really high rate of sporulation is typical of a susceptible reaction where lesions on the upper surface of the leaf are tan (Figure 1B). Plants classified as resistant developed a dark, reddish-brown lesion with few or no spores (Figure 1C) (Hartwig and Bromfield, 1983). Figure 2 from Hahn (2000) depicted the entire soybean rust life cycle.
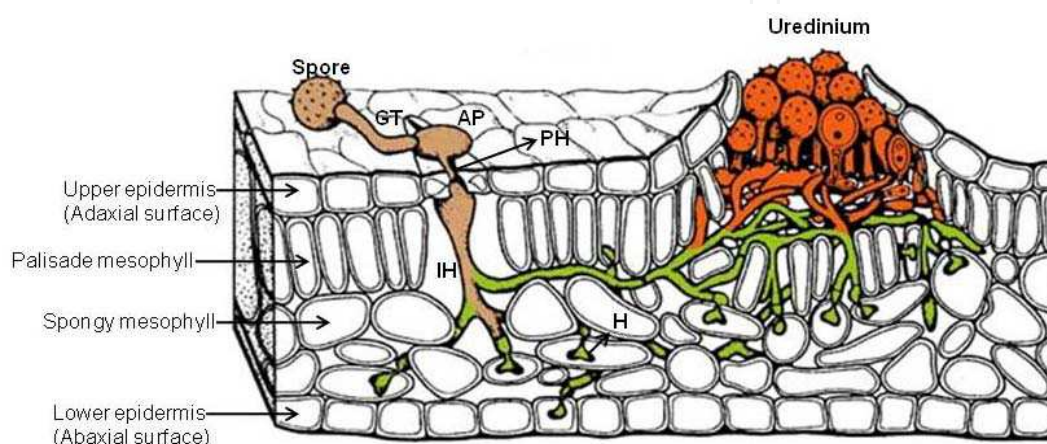

Fig. 2. Internal structure of a typical dicotyledon leaf showing the different cell layers and infection by a rust fungus. GT, germ tube; AP, appressorium; PH, penetration hyphae; IH, infection hyphae; H, haustorium. Schema was taken from Hahn (2000).

1.2 Preventing soybean rust
1.2.1 Fungicides application
Cultural practices such as planting date, row width, and crop rotation sequences have little or no effect on soybean rust, so fungicides are the only option for managing soybean rust until disease-resistant varieties are developed (Shaner et al. 2005). However, there are currently only four chemicals fully registered by the U.S. Environmental Protection Agency for foliar application on soybean. Fungicide application also is expensive and does not always control the pathogen from flowering through pod fill. Timing of foliar fungicide applications often is the key factor that determines success or failure to control fungal plant pathogens. For soybean, fungicide timing has been shown to be critical for the control of some foliar diseases. For example, the control of frogeye leaf spot of soybean (*Cercospora sojina*) varied with applications of benomyl at different reproductive growth stages of the crop (Akem, 1995). To manage soybean rust with fungicides, three strategies include applying fungicides in a predetermined calendar-based schedule (Levy, 2005; Miles et al., 2007), scouting and applying fungicide after first detection of soybean rust, and utilizing a forecast system that monitors disease development in areas that are potential inoculum sources and applying fungicides ahead of a predicted deposition of spores. A calendar-based program with two or three applications provides the greatest level of yield protection; the crop is protected from flowering through grain fill; however, this may result in

unnecessary fungicide applications that increase production costs and may have unforeseen consequences due to activity on non target fungi. Since the appearance of SR in the U.S. continental, studies were mostly conducted in the aim of finding the best fungicide, the right application time and the best way to do it (Mueller et al., 2009; Zhu, 2008; Miles et al., 2007).

1.2.2 Resistance gene discovery

Until now, studies have also been focused on genetic approaches to localize quantitative trait loci (QTLs) on different genotypes of soybean and associate those to resistance soybean genes against soybean rust. Scientists have screened over sixteen hundred soybean accessions for resistance or tolerance to soybean rust (SR) (Miles et al., 2006 and 2008). Five resistance loci have been found (*Rpp1-Rpp5*) in five different accessions. Four have been known since at least the mid-1980s; *Rpp1* (McLean & Byth, 1980), *Rpp2* and *Rpp3* (Hartwig & Bromfield, 1983) and *Rpp4* (Hartwig, 1986). More recently, a new dominant resistance allele, *Rpp5*, was reported by Garcia et al. (2008) and a recessive allele as well (Calvo et al., 2008). Other recent research identified a new allele (*Rpp1b*, Chakraborty et al., 2009) of a known gene. One more rust resistance gene, *Rpp?*, was identified and mapped by Monteros et al. (2007). However, none are present in soybean cultivars commercially grown in the United States. Moreover, there are many isolates of *P. pachyrhizi* and genes conferring resistance to all isolates have not been found which mean that efforts need to be concentrated on finding alternative control measures. From this perspective, understanding the molecular mechanism of the infection process of rust fungi is critical for developing resistant soybean using biotechnology.

1.3 Understand soybean-soybean rust interaction

Only few molecular and biological analyses have been done on *P. pachyrhizi* and on the interaction between *P. pachyrhizi* and its soybean host. One study analyzed gene expression within *P. pachyrhizi* germinating spores (Posada-Buitrago and Frederick, 2005). Four hundred eighty-eight unique expressed sequence tags (ESTs) were generated. One hundred eighty-nine of these ESTs showed significant similarities (e-value $\leq 10^{-5}$) to sequences deposited in the NCBI non redundant protein database. This represents only 39% of the entire library. This highlights the scarcity of genomic information available from pathogenic fungi at that time. These genes were assigned putative roles in many different functional categories. Gene encoding a protein similar to the dehydroshikimate dehydrogenase involved in shikimate pathway leading to the biosynthesis of aromatic amino acids may be use to metabolize metabolic intermediates as alternative carbon sources. Genes encoding homologs of translation initiation factors and elongation factors involved in gene and protein expression suggest that protein synthesis is required for spore germination. Genes encoding a chitin deacetylase, an acetylxylan esterase and a chitin synthase may be involved in cell structure and growth as important for dissolution and formation of the cell wall. Genes encoding some calmodulin kinase homologs involved in cell signaling and cell communication may suggest a calcium-signaling pathway which regulates urediniospore germination as seen in other filamentous fungi. Approximately 30% matched hypothetical proteins or proteins with unknown function. Since then, 47,732 additional ESTs from four different cDNA libraries (uredospores collected from infected soybean leaves, more germinating uredospores on water surface, whole soybean leaf tissue 6-8 days after infection (dai) and, whole soybean leaf tissue 13-15 dai) have been deposited in the NCBI database.

More recently, host gene expression has been studied in whole soybean leaves infected with *P. pachyrhizi* using whole genome Affymetrix microarrays of soybean. Panthee *et al.* (2007) found 112 genes significantly differentially expressed, over 35,611 unigenes present on the array, in soybean plants inoculated with *P. pachyrhizi* 72 h after infection (hai). Forty-six of these genes were up-regulated and 66 were down-regulated. From the up-regulated genes, 14 did not share similarity to known proteins. Most of the up-regulated genes showing similarity to known genes were associated with general defense and stress responses such as salicylic acid (SA)-related protein, heat shock proteins, a leaf senescence-associated receptor-like protein kinase, a glutathione S-transferase, and a chalcone isomerase. Since, they used a soybean cultivar with no known host response to soybean rust, this up-regulation of genes indicated that plants attempt to mount a defense to the pathogen but this was ineffective. There were 20 down-regulated genes that did not share similarity to known proteins. Most of the down-regulated genes encoded peroxidase-related proteins. These proteins catalyzing oxidation-reduction reactions are constitutively expressed in plants and they may be down-regulated by the host during an infection process to divert resources to more pressing needs. van de Mortel *et al.* (2007) added more information by studying soybean response to soybean rust on a susceptible and a resistant host plant at 6, 12, 18, 24, 36, 48, 72, 96, 120 and 168 hai. They found 470 probe sets significantly differentially expressed in both genotypes, whereas 1,046 and 424 probe sets were unique to either the susceptible or the resistant genotypes, respectively. They also described biphasic mRNA changes in soybean in response to SR infection. Within 12 hai, differential gene expression peaked in both genotypes and most (62%) of the common probe sets showed similar expression profiles corresponding basically to nonspecific recognition of SR and activation of basal defense. For example, genes involved in the flavonoid biosynthesis pathway associated to plant defense response were found. Many transcription factors also have been found differentially expressed in a biphasic manner, specifically WRKY transcription factors which may be up or down-regulated. Since WRKY transcription factors change host gene transcription to modulate defenses, this differentially expression suggested complex positive and negative regulation of soybean defense pathways. By 24 hai, mRNA expression of these genes mostly returned to levels found in mock-inoculated plants. This lack of differential expression may come from the low fungal growth at this stage of infection and from the inhibition of the early host responses in the plants. However, a second phase of strong differential gene expression was observed in both genotypes at late stages of infection, which started earlier in the resistant genotype (72 hai) than in the susceptible genotype (96 hai). At these times, only 6% of the common probe sets showed similar expression profiles. Genes encoding components of a sugar transporter superfamily and monosaccharide transporters including a sorbitol transporter as well as genes encoding sugar metabolism enzymes including citrate synthase, isocitrate dehydrogenase, fructose-1,6-bisphosphatase, UDP-arabinose 4-epimerase, and trehalose-6-phosphate synthase have been mostly found differentially expressed in the susceptible genotype and may help to provide nutrients to support fungal infection.

Soria-Guerra *et al.* (2009) studied gene expression in *Glycine tomentella* along a time-course of infection with SR (12, 24, 48 and 72 hai) during a compatible and an incompatible interaction using a 70-mer long-oligo soybean microarray representing about 38,400 genes covering wide developmental stages and physiological conditions. This wild perennial soybean species, as well as others (*G. tabacina* and *G. argyrea*) has been reported as resistant to SR. A total of 1,342 genes were found to be differentially expressed at four time-points in resistant

and susceptible genotypes. About 70% were up-regulated while about 30% were down-regulated at all time-points. As previous studies, most of the up-regulated genes belonged to those with metabolic and defense-related functions in both genotypes, and included enzymes encoding phenylalanine ammonia-lyase involved in phenylpropanoid metabolism, chalcone synthase involved in flavonoid biosynthesis, 4-coumarate-CoA ligase involved in phenylpropanoid metabolism as well as jasmonic acid biosynthesis and lipoxygenases involved in jasmonic acid biosynthesis which increased in expression at 12hai in the resistant and at 24hai in the susceptible reaction. They also found pathogenesis-related genes associated with the development of systemic acquired resistance and encoding anti-microbial proteins up-regulated in the resistant genotypes and down-regulated in the susceptible up to 48hai. Genes involved in cellular communication increased in transcript abundance at all first three time-points in both genotypes has been observed. This included genes encoding protein kinases and zinc fingers all related to the establishment of innate immune response. Also consistent with previous studies, many WRKY transcription factors have been found basically up-regulated later in the susceptible genotype then in the resistant one. Most of the up-regulated genes belonged to the resistant genotype while the down-regulated genes belonged to the susceptible genotype. Overall, all these studies were focusing on gene involved in direct defense response while many more genes encoding proteins involved in amino acid metabolism, nucleotide metabolism or protein destination may be targeted to disturb fungal growth.

Based on what has already been observed about differences between gene expression in infected areas and non infected area on a single infected leaf in regard to photosynthesis (Scholes and Farrar, 1985; Buchanan et al., 1981), we can argue that avoiding background coming from none infected area may provide a better idea about what is really happening at the infection site of the plant in response to the pathogen. Laser capture microdissection (LCM) is a useful tool for isolating infected cells from non-infected cells. Also, the limited number of studies on pathogen infection structures comes from the difficulty in isolating infection structures from the plant. As an obligate biotroph, the fungus cannot be grown on culture medium; it needs live material in which it can proliferate and it can be difficult to separate fungal cells from host cells. LCM can be used to isolate specific fungal infection structures and minimize plant background. LCM has been used relatively recently in plant research to study gene expression in different plant tissues (Kerk et al., 2003) and also to study the interaction between root-knot nematodes, *Meloidogyne* spp., and their host *Lycopersicon esculentum* (Ramsay et al., 2004). An extensive study has been done on the interaction of the soybean cyst nematode, *Heterodera glycines*, with soybean plants using LCM, wherein nematode feeding sites (syncytia) were isolated from soybean roots. Expression levels in those roots were determined using EST and microarray analysis (Klink et al., 2007, 2010). van Driel *et al.* (2007) succeeded in isolating septa from hyphae of the filamentous basidiomcyete *Rhizoctonia solani* grown on an artificial medium. Also, Tang *et al.* (2006) succeeded in isolating individual maize stalk cells associated with *Colletotrichum graminicola* hyphae at an early stage of infection and they identified a number of fungal transcripts associated with early infection of stalk parenchyma by microarray analysis. Kankanala (2007) used positioning ablation laser microdissection (PALM) technology and succeeded to isolate and purify biotrophic invasive hyphae of *Magnaporthe oryzae* 34 hai of rice plants. Both up and down-regulated genes were identified in invasive hypha relative to mycelium, grown in 3 g yeast extract, 3 g casamino acids, 3 g sucrose liquid medium, using *M. oryzae* microarrays.

The main objectives of our study was to improve understanding of SR infection on soybean plants by analyzing (i) the gene expression profile in one of the latest fungal infection structures, the uredinium, and (ii) the soybean genes expression pattern at late stage of infection (240 hai). To achieve these objectives, working on a susceptible genotype, we first constructed a cDNA library from selected uredinium cells of soybean leaves infected with *P. pachyrhizi* isolated using LCM. Resulting sequences allowed identification of specific fungal genes expressed during the infection process. Second, we isolated soybean mesophyll and palisade layer cells using LCM based on morphological changes in the tissues. RNA was extracted from these cells; then cDNA was synthesized, fluorescently labeled and hybridized to a soybean GeneChip® containing 37,744 *Glycine max* probe sets. Microarray results identified sets of genes whose expression patterns show significant alterations in SR inoculated plants. Third, the utilization of a deep sequencing Solexa/Illumina platform on the mesophyll/palisade layer cell samples was tested to analyze the potential offered by this new technology to identify more fungal genes as well as more soybean genes involved in the infection process. Genes identified as involved in the infection process may be used to broaden soybean resistance to *P. pachyrhizi*.

2. Identification of SR genes involved in soybean infection

2.1 Methodology

A *Phakopsora pachyrhizi* isolate (MS06-1) was obtained from urediniospores harvested from field-collected kudzu leaves in Jefferson County, Mississippi in August 2006 and its identity was confirmed by microscopy, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) as previously described (Li et al., 2007). Urediniospores were increased on a susceptible soybean cultivar, Williams 82 (Hyten et al., 2009) in the Stoneville Research Quarantine Facility in Mississippi. The isolate was then purified by picking up a single uredinium using a fine needle under an Olympus SZX12 dissecting microscope, and reinoculating it on leaves of Williams 82. Urediniospores from this purified culture were harvested using a Cyclone Surface Sampler (Burkard Manufacturing Co. Ltd) connected to a vacuum pump, beginning 240 to 336 hai and continuing at weekly intervals.

Inoculum was prepared using freshly collected urediniospores from Williams 82. Spore suspensions were filtered through a 100-μm cell strainer to remove any debris and clumps of urediniospores. Urediniospores were diluted to a final concentration of 1.1×10^5 spores/mL. Three plants per 10 cm-pot with three replicates (pots) were inoculated using 3-week-old seedlings of Williams 82. Inoculant was applied with a Preval sprayer (Younkers) at rate of one milliliter of spore suspension per plant. The same solution minus spores was used for a mock inoculation on three pots of plants to monitor the infection. After inoculation, plants were placed in a dew chamber in the dark at 22°C overnight (approximately 16 h) and then moved to Conviron growth chambers where temperatures were maintained at 23°C during the day and 20°C at night under a 16-h photoperiod with a light intensity of 280 μEm^{-2} s^{-1}. SR inoculated and mock-inoculated plants were kept in two different growth chambers.

Soybean leaf tissue was harvested 240 hai. The leaf tissue was cut into 1 cm square pieces and vacuum-infiltrated with Farmer's solution (FS) composed of 75% ethanol and 25% acetic acid (Sass, 1958) at room temperature for 1 h. Fresh FS was added to the samples and tissue was subjected to an incubation step of 12 h at 4°C. The fixative was removed, and leaves were dehydrated with a graded ethanol series (75%, 85%, 100%, 100%), 30 min each. Ethanol

was replaced with 1:1 xylene:ethanol for 30 min, followed by three, 100% xylene incubations (30 min each). Xylene was then replaced with paraffin by placing the specimens into a 58°C oven and infiltrating the leaves sequentially in 25, 50 and 75% (1x) and 100% (3x) Paraplast + tissue embedding medium (Tyco Healthcare Group LP) in each step for 3 h. Tissue was cast and mounted for sectioning. Serial sections of leaves were made on an American Optical 820 microtome (American Optical Co.) at a section thickness of 10 μm. Serial sections were placed onto a pool of RNAse-free dH$_2$O, on Leica PEN-Membrane 2.0 uM membrane slides.

About five million square microns surface of uredinium sites from infected leaves (Figure 3A-B) were collected by LCM 240 hai using a Leica ASLMD microscope (Leica®).

RNA was extracted and isolated from LCM-collected uredinia cells using an isolation Kit specific for small RNA quantity (Arcturus Bioscience Inc.). A unidirectional cDNA library was constructed using an improved method for library construction from microdissected cells (Peterson et al., 1998).

Sequencing was conducted using BigDye 3.1 chemistry (Applied Biosystems). Reactions were analyzed on an Applied Biosystems 3730XL sequencer. Sequence base-calling was performed using PHRED (http://www.phrap.org) (Ewing and Green, 1998; Ewing et al., 1998) with a quality score of 20 (99% confidence). Vector and low quality sequences were removed using Lucy (Chou and Holmes, 2001). The trimmed sequences were assembled into consensus sequences using CAP3 (Huang and Madan, 1999) to reduce redundancies.
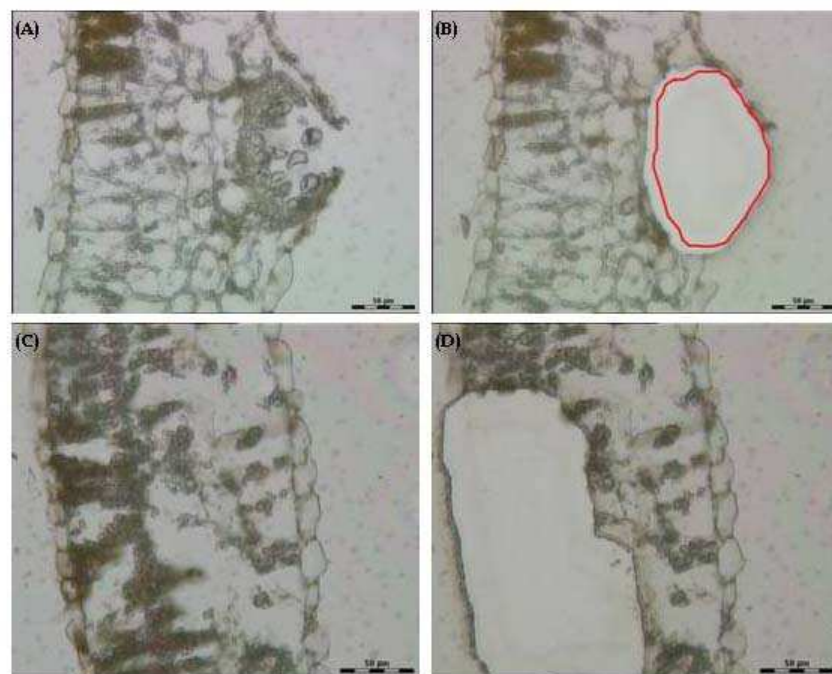

Fig. 3. LCM of uredinium site from soybean leaf 10dai with soybean rust. (A) Longitudinal section of soybean leaf with a uredinium on the lower leaf surface before LCM visualized at 40X magnification. (B) The same section after LCM with the microdissected uredinium region absent from the section. (C) Longitudinal section of soybean leaf showing brown coloration inside the mesophyll/palisade cells before LCM visualized at 40X. (D) The same section after LCM showing that the microdissected palisade region is absent from the section. Scale bar represents 50 μm.

The data presented here represents the later assembly process and the resulting contigs and singletons were designated as unisequences. Appropriate Paracel Blast (Striking Development, http://www.paracel.com/) algorithms were used to analyze the contigs and singelton sequences against the following databases: NCBI Vector (current to 2006), NCBI nr nucleotide (current to December, 2007), NCBI nr protein (current to December, 2007), NCBI est-others (current to December, 2007), NCBI *Glycine max* trace (ftp://ftp.ncbi.nih.gov/pub/TraceDB/glycine_max current to December 2007), NCBI *Phakopsora pachyrhizi* trace (ftp://ftp.ncbi.nih.gov/pub/TraceDB/phakopsora_pachyrhizi) current to 16 June 2004).

2.2 Results and discussion

Of the five cDNA libraries from *P. pachyrhizi* represented in NCBI public databases, all came from either uredospores germinating on water or from non-germinating or whole infected leaf tissue. This work represents the first LCM-derived cDNA library specifically enriched from the uredinium stage of SR development. This reddish or black pustule-like structure comprises the uredospores-producing fruiting body that is formed on the tissue of infected plants. At 240 hai the uredinium contains uredospores at all developmental stages, not only the mature stage. It also contains other kinds of fungal cells including peripheral paraphyses (Berndt, 2005; Hernandez et al., 2003). Unlike germinating spores, which are rapidly growing and preparing to enter the host, uredinia produce spores for dispersal and survival of a new generation. Hence, genes involved in spore production, spore development, and genes expressed in mature uredospores can be found.

A total of 925 clones were sequenced from both ends of the cDNA inserts. The generated sequences resulted in an average of 568 nucleotides readable after editing. The term unisequences is used to identify the assembled products regardless if they are represented by contigs or singletons. A total of 130 unisequences from CAP3 were identified of which 70 were singletons and 60 were contig assemblies representing multiple clones at frequencies ranging from 2 to 431. Over the 130 unisequences identified, 117 displayed similarity to sequences available in NCBI *Phakopsora pachyrhizi* trace, EST-others and nucleotide databases (nucleotide blast; cut-off set e-value $\leq 10^{-5}$). Thirteen unisequences displayed similarity to sequences from *Glycine max* trace, EST-others and nucleotide databases (nucleotide blast; cut-off set e-value $\leq 10^{-5}$). Forty unisequences with significant hits to the NCBI nr protein database (blastx; cut-off set e-value $\leq 10^{-5}$) were grouped according to their putative function (Table 1). Approximately half (21) of those unisequences correspond to hypothetical proteins of unknown function (Table 1). For deduced protein sequences that lacked significant similarity to proteins or sequence translations from GenBank, an extra comparison was done against the InterPro database of protein domains and functional sites (http://www.ebi.ac.uk/Tools/InterProScan/), revealing the presence of conserved domains in several unisequences (Table 1). The expression of seventeen unisequences out of the forty with significant homology, chosen for their high homology with proteins or conserved protein domains that might be involved in the infection process, or their high homology with other ESTs found in a uredospore library of *P. pachyrhizi* isolate TW 72-1, have been analyzed using quantitative PCR at eight additional time-points (time-zero, 2, 7, 20, 24, 48, 96 and 168 hai) (data not showed).

Unisequence	Accession No.	Description	Species	e-value (10^{-x})	Clone frequency
Metabolism					
Singleton61	EAY48797	TonB-dependent vitamin B12 receptor	*Escherichia coli*	-92	1
Energy					
Contig6	XP_567378	Isocitrate dehydrogenase (NAD+)	*Cryptococcus neoformans* var. *neoformans*	-57	2
Cell growth, cell division and DNA synthesis					
Singleton30	EDO65226	activator 1 37 kDa subunit	*Neurospora crassa*	-70	1
Transcription					
Contig58	ABN66688	Histone acetyltransferase SAGA	*Pichia stipitis*	-17	2
Protein synthesis					
Contig 24	AAA84030	Ribosomal protein S17	*Arabidopsis thaliana*	-25	12
Protein destination					
Singleton48	EAL73572	dolichyl-phosphate-mannose alpha-1,3-mannosyltransferase	*Dictyostelium discoideum*	-10	1
Contig 38	BAB33421	Putative senescence-associated protein	*Pisum sativum*	-35	3
Cellular biogenesis					
Contig54	AAW43806	Nucleosome assembly protein	*C. neoformans var. neoformans*	-35	97
Singleton1	ABH11422	Peroxin16	*Penicillium chrysogenum*	-23	1
Cellular communication/signal transduction					
Contig10	XP_001541001	Serine/threonine-protein phosphatase PP1	*Ajellomyces capsulatus*	-56	11
Cell rescue, defense, cell death and ageing					
Singleton44	EAW24135	UV excision repair protein (RadW)	*Neosartorya fischeri*	-12	1
Cellular organization					
Singleton22	BAD90781	Histone 3	*Conocephalum conicum*	-49	1
Transposon, insertion sequence and plasmid proteins					
Contig17	AAD03794	Tc1-like transposase	*Anopheles gambiae*	-14	8
Contig27	ABG66123	Retrotransposon protein	*Oryza sativa*	-05	3

Unisequence	Accession No.	Description	Species	e-value (10^{-x})	Clone frequency
Contig33	EAL59155	Transposase	*Wolbachia* endosymbiont	-36	3
Singleton60					1
Contig17	AAD03794	Tc1-like transposase	*Anopheles gambiae*	-14	8
Unclassified					
Singleton49	ABX34586	putative phage major head protein	*Delftia acidovorans*	-57	1
Singleton52	AAZ28943	polyprotein	*Phanerochaete chrysosporium*	-11	1
Contig1	EDO03956	Hypothetical protein SS1G_06437	*Sclerotinia sclerotiorum*	-06	11
Contig2	EAL20633	Hypothetical protein CNBE2980	*C. neoformans var. neoformans*	-05	27
Contig3	CAN72993	Hypothetical protein	*Vitis vinifera*	-06	6
Contig7[a]	EAU89149	Chromo-domain-like	*Coprinopsis cinerea okayama*	-38	6
Contig9[a]	EAU89356	Transposase Tc1/Tc3	*C. cinerea okayama*	-21	6
Contig28[a]	EAU80277	DNA/RNA polymerase	*C. cinerea okayama*	-43	3
Contig35	EAU84377	Hypothetical protein CC1G_01373	*C. cinerea okayama*	-24	2
Contig39[a]	EDN29539	LDOC1 related	*Botryotinia fuckeliana*	-14	2
Contig42[a]	EDJ99713	Retrotransposon gag protein	*Magnaporthe grisea*	-15	3
Contig43	CAK45797	Hypothetical protein An08g11260	*Aspergillus niger*	-07	31
Contig44[a]	EAU87561	Signal peptide	*C. cinerea okayama*	-09	3
Singleton36[a]				-06	1
Singleton56[a]				-20	1
Contig46	EDR00494	Predicted protein	*Laccaria bicolor*	-09	6
Singleton10	XP_001501620	Hypothetical protein	*Equus caballus*	-07	1
Singleton12	CAN73669	Hypothetical protein	*V. vinifera*	-13	1

Unisequence	Accession No.	Description	Species	e-value (10^{-x})	Clone frequency
Singleton13	EDP41532	hypothetical protein MGL_4081	*Malassezia globosa*	-08	1
Singleton14[a]	EAU91239	Phospholipase-related	*C. cinerea okayama*	-19	1
Singleton28	EAK86148	hypothetical protein UM04768.1	*Ustilago maydis*	-20	1
Singleton55	ABQ37728	hypothetical protein BBta_5781	*Bradyrhizobium sp.*	-08	1
Singleton67[a]	EDN30101	Signal peptide	*B. fuckeliana*	-11	1
Singleton68	EDN03050	predicted protein	*A. capsulatus*	-12	1

Table 1. Unisequences displaying similarity (e-value ≤ 10^{-5}) to proteins in the nonredundant protein NCBI database, grouped in functional categories. [a] Unisequence displaying similarity to an hypothetical protein but displayed similarity to a specific conserved domain.

Libraries constructed from whole infected plants typically contain many more plant genes than fungal genes. By using LCM to collect infection sites, we reduced the number of plant ESTs versus fungal ESTs generated from infected leaf tissue. Sixty-three percent of the unisequences shared similarity to proteins from fungi (including yeast) while 16% encoded plant protein sequences. The other 21% encoded protein sequences with higher similarity to other organisms, including bacteria, viruses, insects and other invertebrates. These percentages are similar to results obtained by other research groups studying filamentous fungi (Lee et al., 2002a; Trail et al., 2003).

Twenty-nine of the 40 unisequences presented in Table 1 are novel *P. pachyhrizi* sequences. While eleven unisequences corresponded to existing *P. pachyhrizi* sequences, none matched the sequences described by Posada-Buitrago and Frederick (2005) from a cDNA library from mature spores. Considering that this library is one of the few *P. pachyrhizi* cDNA libraries that have been analyzed thus far, our results demonstrate that a different gene set is expressed in uredinium compared to germinating spores.

The infection process includes three major steps: germination and penetration, proliferation and sporulation (Solomon et al., 2003; Zhang et al., 2008). At 240 hai, sporulation occurs. Identifying genes involved in sporulation events is a necessary first step toward using disruption of these genes as a way to prevent disease dissemination. Several of the unisequences from the uredinium-enriched library were identified as having similarity to proteins or domains that are consistent with functions known to be necessary for sporulation.

Fungi need energy at different stages of development. For aerobic fungi, the oxidation of glucose or fatty acids into the tricarboxylic acid cycle can provide up to 38 ATP molecules. One enzyme involved in this cycle is isocitrate dehydrogenase (Contig6), which catalyzes the oxidative decarboxylation of isocitrate to produce α-ketoglutarate and carbon dioxide while it converts the NAD^+ coenzyme into NADH. NADH can interact with the electron transport chain, resulting in the production of ATP (Isaac, 1992). Contig6, which showed significant sequence similarity to isocitrate dehydrogenases, was expressed most highly at 20, 48 and 240 hai. In rust fungi, the initial haustorial mother cell typically forms between 24

and 48hai, consistent with the time frame of Contig6 expression. Jakupović *et al.* (2006) found many different genes involve in energy production expressed in a haustorium cDNA library. But at 240 hai, the uredinium needs energy to produce uredospores.

Reversible protein phosphorylation is a key mechanism to modulate the activity of proteins. It plays a central role in a variety of types of cellular regulation, including control of metabolism, cell cycle, cell proliferation, and differentiation (Stark, 1996). Protein phosphorylation can be conducted by enzymes such as protein kinase C, cyclic AMP (cAMP)-dependent protein kinase, mitogen-activated protein (MAP) kinase, and type 1, 2A, and 2B serine/threonine phosphatases. Clones representing transcripts of some of these enzymes have been found in multiple cDNA libraries from filamentous fungi (Sacadura and Saville, 2003; Zhang et al., 2008). Our results indicate that Contig10 has similarity with a serine/threonine protein phosphatase PP1 from *Ajellomyces capsulatus*, while Contig41 encodes a serine/threonine protein kinase domain. Formation and differentiation of uredospores within the uredinium requires several proteins that are found during normal growth (Hall et al., 1999; Lengeler et al., 2000). Since protein phosphorylation is involved in many differentiation processes, PP1 might be one of the important enzymes playing a role in spore differentiation at 240 hai. Expression of Contig10 at 2hai might be involved in a quick cell proliferation at the beginning of the infection.

The DNA sequence of Contig13 is 100% identical to an EST found in *P. pachyrhizi* TW 72-1 germinating spores, but the function of that EST is unknown. However, this unisequence is expressed in the early steps of the infection process (between 2 and 24 hai). At 240 hai, this unisequence is expressed at higher levels than at the beginning of the infection and higher than the other unisequences tested by quantitative PCR. It would be expected that all of these genes would be strongly expressed at 240 hai, because they were derived from the uredinia collected at that time. Some of the genes we identified not only are expressed at 240 hai but at other time points as well, indicating that they have important roles during infection and growth, as well as during spore development. van de Mortel *et al.* (2007) found a distinct biphasic change in mRNA expression in soybean in response to SR. If this kind of response can be observed in soybean, it is possible that a similar response also occurs in the pathogen. One possible explanation is that at the beginning of infection, the pathogen concentrates its energy and effort on infecting the leaf, and many different genes are needed at this stage. Thus, this step may represent the first burst of gene expression. The host plant responds to this infection by its own first burst of gene expression. Then the pathogen proliferates within the plant leaf, coinciding with a second burst of gene expression. Therefore, Contig13 might represent an important gene involved in many steps during fungal infection, proliferation and reproduction.

Contig39, which contains a domain similar to LDOC1 domain, is expressed in the plant before the inoculation. LDOC1 domain in mammalian cells was found to be involved in negative regulation of cell proliferation and in differentiation of cancer cells (Nagasaki et al., 1999). The presence of this kind of domain in plants or fungi has not been described previously, but its expression in our library along with its known function in mammalian cells attracts attention.

Protein secretion is a critical process in the life cycle of fungi, and filamentous species display a natural ability to secrete large amounts of native proteins. For example, many Avr proteins are secreted from extracellular fungal pathogens, as occurs with bacterial Avr proteins (Luderer et al., 2002; Rep et al., 2004). In biotrophic fungi such as rust and mildew, virulence and avirulence factors and pathogenicity mechanisms are difficult to study. The

first rust Avr protein identified, AvrL567 of the flax rust *Melampsora lini*, contains a predicted signal peptide, suggesting that it is secreted (Dodds et al., 2004). In the oomycetes, a specific conserved motif has been found in different secreted protein sequences (Ellis et al., 2006). Although many of our unisequences appear to encode a signal peptide, this "RxLR" motif was not observed in any of our unisequences. Furthermore, all of the unisequences containing signal peptides that were analyzed by quantitative PCR showed specific expression late in the infection process, not earlier.

A phospholipase-related domain was encoded by Singleton14. Phospholipase proteins are secreted proteins, and in *Candida albicans* and *Cryptococcus neoformans*, phospholipase B protein has been identified as the virulence factor (Cox et al., 2001; Ghannoum, 2000). Because the expression of this unisequence was limited to 240 hai and rather low, the significance of the phospholipase domain is not clear.

Contig50 was the most abundant sequence in our library, comprises of 431 ESTs. Contig11 was third in abundance, containing 63 sequences. Neither of these contigs showed similarity with protein or *P. pachyrhizi* sequences in NCBI databases. While they were highly represented in our library, each was expressed only at 240 hai.

Transposable elements (TE) have been found and studied in filamentous fungi (Daboussy and Capy, 2003; Kempken and Kück, 1998). Our results show several unisequences (Contig9, Contig17, Contig27, Contig33 and Singleton60) with high homology to transposases in the *Tc1/mariner* family or containing stretches of sequence similar to a transposase Tc1/Tc3 domain. Since most of the similarity was with genes encoding TE from flies, plants and bacteria, we don't know if these expressed genes were coming from the rust side or the plant side. We know that plant genome stability is affected by abiotic stresses (Lebel et al., 1993; Puchta et al., 1995) but there are only few evidences about it during biotic stresses. Lucht et al. (2002) found that there is an increase in somatic recombination frequency in Arabidopsis following the infection with the oomycete pathogen *Peronospora parasitica*. With these results, they were tempted to speculate that somatic recombination events stimulates by pathogen stress might be involved in the evolution of plant R-gene clusters and new pathogen specificities. Since only few soybean cultivars have resistance to SR isolates, the TEs identified in our experiment may play some role in the lack of resistance in soybean plants. A similar phenomenon was observed in rice, where Pot3 transposon introduced into the promoter of *AVR-Pita*, an avirulence gene of *Magnaporthe grisea*, caused the gain of virulence toward Yashiro-mochi, a rice cultivar containing the disease resistance gene *Pi-ta* (Kang et al., 2001).

3. Identification of soybean genes involved in response to SR infection

3.1 Methodology

A microarray study was performed to identify soybean genes expressed in response to SR. Trifoliate leaves of soybean cv. Williams 82 inoculated with *P. pachyrhizi* isolate MS06-1 240 hai showed brown coloration in the mesophyll/palisade cells layer most of the time close to an uredinium but sometimes far from an uredinium (Figure 3C-D). Three million square microns of mesophyll/palisade cells showing this brown coloration were collected by LCM as well as 3,000,000 µm² of noninfected mesophyll/palisade cells serving as control. RNA was extracted from each 1,000,000 µm² of mesophyll/palisade cells (three replicates from each infected and noninfected samples) by using the same technique used to extracted RNA from uredinium-enriched samples. RNA was amplified to increase starting material. The

GeneChip® Soybean Genome Array (Affymetrix®) was used for the microarray analyses. The array contains 37,744 *G. max* probe sets (35,611 transcripts). Details of the GeneChip® Soybean Genome Array can be obtained at (http://www.affymetrix.com/index.affx).

3.2 Results and discussion

A total of 2,020 genes were found to be significantly differentially expressed (fold-changes of ≥ 2.0 and p-values ≤ 0.05 and a false-discovery rate of 5%) in mesophyll/palisade cells infected by SR, as compare to non-infected control plants. Out of the 2,020 differentially expressed genes, 380 were induced, and 1,640 were suppressed.

There were 1,442 down-regulated genes identified that share similarity with genes encoding known proteins, while 60 down-regulated genes share similarity with unknown proteins and 138 genes share no similarity (*e*-value $\leq 10^{-2}$). All annotations were obtained by performing BLASTX from the Affymetrix ID accession available at http://affymetrix.com/index.affx. The fifty most down-regulated genes are listed in Table 2. Most of the down-regulated genes with similarity to genes encoding known proteins were included in the metabolism and energy functional categories (Figure 4A).

During an infection, a lot of changes occur in the host plant tissues (Hoch, 1993). The first major change consists of a decrease in the rate of photosynthesis correlated with an increase in the area of infected tissues. When broad beans are infected by rust and sugar beets by powdery mildew (Farrar and Lewis, 1987), chloroplasts lose their structural integrity due to the pathogen infection. This change in the chloroplast ultrastructure corresponds to a reduction in the capacity for sucrose production. Early in the infection process of leaves by rust, the photosynthetic rate is unaltered; after sporulation there is a decrease in the ability of the plant to fix carbon, which seems to be related to the destruction of the chloroplasts, the degradation and the loss of chlorophyll concentration.

The infection of the plant causes a block in the non-cyclic electron transport chain by reducing the amount of cytochromes without affecting the integrity of the photosystems I and II. Indeed, our results show that photosynthesis in soybean leaves is highly affected 240 hai by SR as well as carbon fixation. Numerous genes encoding enzymes involved in photosynthesis are suppressed compared to the uninfected plant including genes encoding chlorophyll A-B binding proteins (LHCA2, LHCB4.2, LHCB4.3, CAB, LHCA3.1, LHB1B1, LHB1B2, LHCB2.1, LHCB2.3, LHCB3, etc), photosystem I reaction center (subunit II, III, psaK, PSI-N, V, VI, XI) and photosystem II proteins, ferredoxin proteins, and fructose-bisphosphate aldolase. These results are in agreement with Polesani *et al.* (2008), who studied transcriptome changes in grapevine infected by *Plasmopara viticola* causing mildew. The most striking transcriptional down-regulation in grape leaves infected with mildew was observed in genes involved in photosynthesis e.g. chlorophyll a-b binding proteins and photosystem components, consistent with a measurable reduction in chlorophyll content during pathogenesis. Transcriptional down-regulation of photosynthesis-related genes has been reported previously also during compatible interactions between potato and *P. infestans* (Moy et al., 2004) and between soybean and *P. sojae* (Restrepo et al., 2005).

The second major change consists of the uncoupling of oxidative phosphorylation in the chloroplast. This uncoupling prevents ATP synthesis via the electron transfer chain and favors the accumulation of ADP and the increase in rate of oxygen uptake. This increase in the rate of respiration is probably due to the presence of respiring fungal tissues. Such increased respiratory levels lead to the rapid depletion of the plants carbohydrate reserves. Since there is less carbohydrate available for production of ATP molecules, an increase in

Probe set	Gene Annotation	Fold change	p Value (-10^x)
Down-regulated			
Gma.10892.5.S1_at	Carbonic anhydrase	-929,3	-05
Gma.5294.1.S1_at	gibberellin-regulated family protein	-624,9	-04
Gma.1379.2.A1_at	Gonadotropin, beta chain; Gibberellin regulated protein	-499,6	-07
Gma.10892.1.S1_a_at	Carbonic anhydrase	-498,9	-03
Gma.1201.1.A1_at	Hypothetical protein	-475,5	-04
Gma.3304.1.S1_at	Plant lipid transfer/seed storage/trypsin-alpha amylase inhibitor	-391,4	-05
Gma.11116.4.S1_at	Major intrinsic protein	-387,0	-03
Gma.3241.1.S1_a_at	Germin-like protein	-363,6	-04
Gma.11116.2.S1_at	Expressed protein	-342,6	-05
Gma.3208.2.S1_a_at	Oxalic acid oxidase	-241,5	-05
Gma.15007.1.A1_s_at	Ferredoxin [2Fe-2S], plant	-237,0	-03
Gma.15091.2.S1_at	unknown protein	-232,6	-04
Gma.10591.2.S1_at	Glycine cleavage system H protein, mitochondrial precursor	-231,7	-05
Gma.1355.2.S1_s_at	ribosomal protein L17 family protein	-230,5	-06
Gma.4575.1.S1_at	Fructose-bisphosphate aldolase	-226,4	-05
Gma.11254.2.S1_at	SAH7 protein	-224,2	-05
Gma.5294.1.S1_s_at	gibberellin-regulated family protein	-214,0	-03
Gma.1992.1.S1_at	Hypothetical protein	-204,3	-04
Gma.3161.1.S1_at	Glutamine synthetase	-187,3	-03
Gma.17554.1.S1_a_at	Light harvesting chlorophyll a /b binding protein of PSII	-186,5	-04
Gma.4385.1.S1_s_at	fasciclin-like arabinogalactan protein FLA2	-183,7	-04
Gma.10620.1.S1_at	NADH-dependent hydroxypyruvate reductase	-181,0	-04
Gma.10151.1.S1_at	thylakoid soluble phosphoprotein	-175,8	-04
Gma.2224.1.S1_s_at	Tubulin beta-1 chain	-175,6	-04
Gma.10771.1.A1_a_at	thiamin biosynthesis protein	-171,0	-05
Gma.15376.1.A1_s_at	Photosystem I reaction center subunit VI, chloroplast precursor	-170,9	-07
Gma.1160.1.S1_at	Ferritin-3, chloroplast precursor	-168,545	-04
Gma.289.1.S1_s_at	Ribulose bisphosphate carboxylase small chains, chloroplast precursor	-161,4	-04
Gma.9720.1.S1_at	Transketolase, C-terminal-like	-155,6	-04
Gma.2503.1.S1_at	NADH-dependent hydroxypyruvate reductase	-151,0	-03
Gma.1955.4.S1_a_at	Chloroplast oxygen-evolving enhancer protein	-148,4	-03
Gma.11116.4.S1_s_at	Major intrinsic protein	-148,1	-04

Probe set	Gene Annotation	Fold change	*p* Value (-10^x)
Gma.10852.2.S1_at	Photosystem I reaction center subunit II, chloroplast precursor	-139,8	-03
Gma.15830.1.S1_s_at	Expressed protein	-137,6	-04
Gma.5785.1.S1_at	endo-1,4-beta-glucanase, / cellulase, putative	-135,8	-05
Gma.1791.1.S1_at	Ferredoxin-B	-135,8	-05
Gma.11034.1.S1_at	Oxygen-evolving enhancer protein 1, chloroplast precursor	-135,6	-02
Gma.289.1.S1_x_at	Ribulose bisphosphate carboxylase small chains, chloroplast precursor	-135,5	-05
Gma.2360.1.S1_at	Light harvesting chlorophyll a /b binding protein of PSII	-134,6	-05
Gma.15462.1.S1_a_at	RNA-binding region RNP-1	-129,8	-03
Gma.3317.2.S1_a_at	peroxiredoxin Q, putative	-128,7	-02
Gma.2360.3.S1_at	Light harvesting chlorophyll a /b binding protein of PSII	-124,5	-03
Gma.16678.1.S1_at	Chloroplast thioredoxin M-type	-124,0	-06
Gma.16829.1.S1_x_at	Nodulin-26	-124,0	-02
Gma.7309.2.S1_s_at	Glycolate oxidase	-122,8	-02
Gma.15620.1.S1_at	50S ribosomal protein L12, chloroplast precursor	-122,1	-04
Gma.15814.1.A1_at	Xyloglucan endotransglycosylase/hydrolase 16 protein	-121,1	-03
Gma.10771.3.S1_x_at	thiamin biosynthesis protein	-118,4	-06
Gma.12822.1.S1_at	cytochrome b6f complex subunit (petM), putative	-117,1	-04
Gma.14123.1.S1_at	Hypothetical protein	-116,1	-04
Up-regulated			
Gma.15636.2.S1_x_at	Hypothetical protein	621,4	-03
Gma.3702.1.S1_at	Endochitinase PR4 precursor	492,6	-05
Gma.17733.1.S1_s_at	Proteinase inhibitor I13, potato inhibitor I	458,2	-03
Gma.6999.2.S1_s_at	Stress-induced protein SAM22	438,6	-02
Gma.2821.1.S1_at	Osmotin	416,8	-04
Gma.6999.1.S1_s_at	Stress-induced protein SAM22	409,0	-02
Gma.3734.1.S1_at	Proteinase inhibitor I13, potato inhibitor I	366,6	-06
Gma.5574.1.S1_s_at	Pleiotropic drug resistance protein 12	335,8	-06
Gma.6999.1.S1_x_at	Stress-induced protein SAM22	220,7	-03
Gma.3713.1.S1_s_at	Aldo/keto reductase AKR	183,8	-04
Gma.2593.1.S1_s_at	Glutathione S-transferase GST 15	182,2	-03
Gma.772.1.S1_at	Hypothetical protein	143,1	-03
Gma.5529.1.S1_at	NAD(P)H-dependent 6'-deoxychalcone synthase	133,8	-04
Gma.12045.1.S1_at	Asparagine synthetase 1	128,3	-04

Probe set	Gene Annotation	Fold change	p Value (-10^x)
Gma.9397.1.S1_at	NHL3 (NDR1/HIN1-like 3)	125,4	-04
Gma.16547.1.S1_at	WRKY86	116,9	-03
Gma.6327.1.S1_s_at	B12D protein	114,5	-04
Gma.5950.1.S1_s_at	Dirigent protein	107,8	-04
Gma.10717.1.S1_a_at	integral membrane family protein	105,5	-03
Gma.2096.1.S1_at	Protein At2g29340	104,2	-03
Gma.3604.1.S1_at	Caffeoyl-CoA O-methyltransferase 1	93,3	-05
Gma.5709.1.S1_at	unknown protein	87,8	-03
Gma.9947.1.S1_at	Hypothetical protein	84,4	-05
Gma.16778.1.S1_at	VQ motif-containing protein	83,1	-04
Gma.744.1.S1_at	WRKY transcription factor 41	77,9	-04
Gma.4185.1.S1_at	ATEXLB1 (EXPANSIN-LIKE B1)	77,8	-04
Gma.1654.1.S1_s_at	Coatomer protein complex, beta prime; beta'-COP protein	74,6	-03
Gma.7559.1.S1_s_at	Polyphenol oxidase	72,6	-05
Gma.16709.1.S1_s_at	Cytochrome P450 monooxygenase CYP82E13	67,8	-05
Gma.15568.1.S1_at	disease resistance-responsive protein-related / dirigent protein-related	66,9	-04
Gma.2821.2.S1_a_at	thaumatin-like protein	66,7	-04
Gma.17802.1.S1_at	UVI1	63,0	-04
Gma.17305.1.S1_at	alpha-hydroxynitrile lyase	62,4	-04
Gma.4375.1.S1_s_at	Cytosolic glutamine synthetase beta2	57,0	-02
Gma.2523.1.S1_s_at	R 14 protein	50,3	-05
Gma.1537.1.S1_at	Vesicle-associated membrane protein 725	50,0	-03
Gma.8331.1.S1_at	Aldehyde dehydrogenase [Vitis	49,6	-05
Gma.15664.1.S1_at	2'-hydroxydihydrodaidzein reductase	49,1	-03
Gma.2821.2.S1_at	thaumatin-like protein	48,6	-03
Gma.7728.1.S1_at	LacZ protein	48,0	-03
Gma.8401.1.A1_at	Cytochrome P450 71D8	48,0	-02
Gma.1748.1.S1_at	NAC domain protein NAC1	47,5	-05
Gma.17594.1.A1_at	F1N19.23	47,1	-04
Gma.2773.2.S1_at	F12P19.3	46,9	-03
Gma.5129.1.S1_at	pyridine nucleotide-disulphide oxidoreductase family protein	46,8	-03
Gma.4483.1.S1_at	AMP-binding protein	46,3	-03
Gma.17929.1.A1_at	Transferase	45,7	-04
Gma.2578.1.S1_at	endo-1,3-beta-glucanase	45,4	-05
Gma.12031.2.S1_x_at	F17O7.4	45,2	-04
Gma.8113.1.A1_at	No apical meristem (NAM) protein-like	44,9	-03

Table 2. List of the fifty most greatly induced and suppressed annotated genes in soybean cv. Williams 82 10 dai by SR (pValue ≤ 0.05).

pentose phosphate metabolism is needed. This is what is seen in some plant-fungal interactions such as rice infected by the sheath blight fungus, *Rhizectonia solani* (Danson et al., 2000). In this case, the PEP carboxylase is down-regulated. However in our experiment, we observed a massive decrease in the abundance of transcripts of genes important in the pentose phosphate metabolism as well as most of the other pathways of carbohydrate metabolism that produce ATP, including the tricarboxylic acid (TCA) cycle, starch and sucrose metabolism, galactose metabolism, inositol phosphate metabolism, glycolysis, and the pentose phosphate pathway. This shutdown was reflected by the down-regulation of many genes involved in carbohydrate metabolism as well as carbon fixation such as genes encoding ribulose-bisphosphate carboxylase, ribulose-phosphate 3-epimerase, transketolase, sedoheptulose-bisphosphatase, fructose-bisphosphate aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. The overlay of all significantly differentially expressed genes on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways has been used to facilitate results interpretation. PAICE, a tool for coloring KEGG pathways given Enzyme Commission (EC) accessions (http://paice.sourceforge.net) has been used to perform this analysis. This bioinformatics software was employed for its ability to handle duplicate gene-copies as well, color genes with large variances between time points, as well as color accessions given fold-change values. Figure 5 represents the pentose phosphate metabolism where enzymes corresponding to encoded genes expressed in our dataset have been colored depending on their expression level. Some of energy required by plants for respiration is probably coming from the conversion of lactate to pyruvate by lactate dehydrogenase. Pyruvate goes to the TCA cycle to produce ATP molecules needed. This alternative way of producing ATP molecules is highlighted in our study by the up-regulation of some enzymes involved in the pyruvate metabolism.

Pentose phosphate metabolism is the main pathway for production of phenolic compounds, which are responsible for the activation of defense mechanisms. Suppression of expression of many genes encoding proteins involved in pentose phosphate pathway, including genes encoding transketolase, ribulose-phosphate 3-epimerase, ribose-phosphate pyrophosphokinase 1 and fructose-bisphosphate aldolase, present in our results suggest that pentose phosphate metabolism is down-regulated during SR infection which agrees with
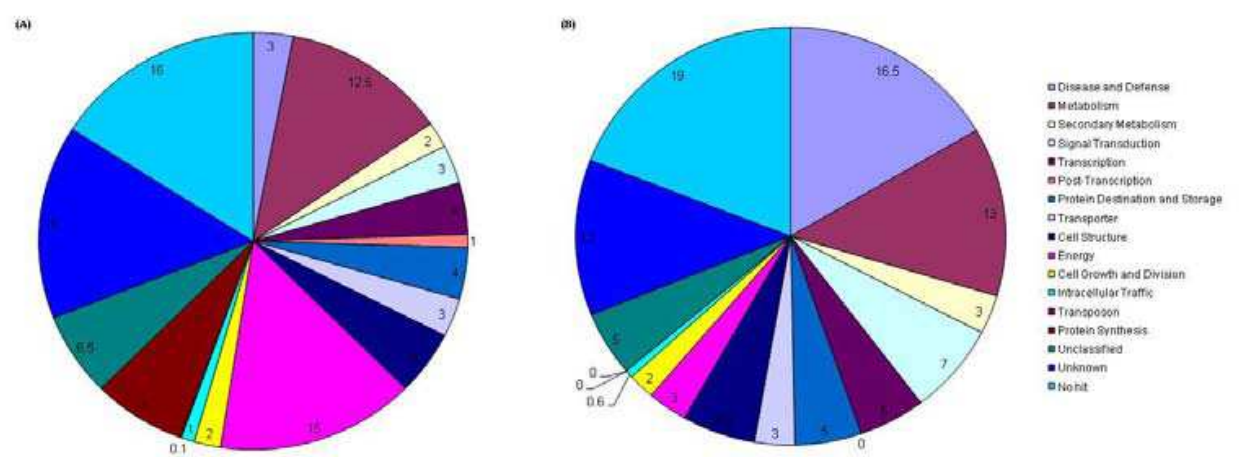

Fig. 4. Functional categorization of the probe sets (a) down- (%) and (b) up-regulated (%) in soybean cultivar Williams 82 infected by SR 10dai.
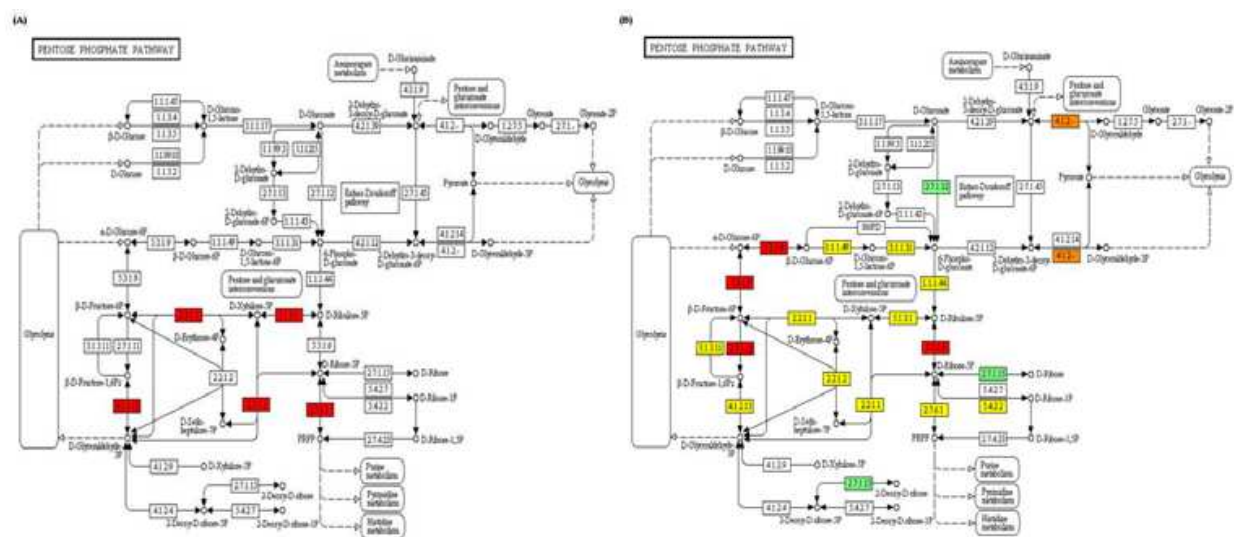
Fig. 5. Expression levels of genes encoding enzymes overlaid on the pentose phosphate pathway. The expression level for each gene encoding enzymes is associated with a specific color. Enzymes colored in red are down-regulated, the ones colored in green are up-regulated (regular red or green when expression falls in plus or minus 25% the mean expression value range, dark red or green when expression is greater than 25% of the mean expression value and light red or green when expression is lower than 25% of the mean expression value), the ones colored in orange are extremely down-regulated while the ones colored in aqua are extremely up-regulated and finally the ones colored in yellow indicates that this specific gene encodes for different forms of the same enzyme and those different copies are differentially regulated. The color code is the same for fig.6.

the fact that susceptible plant don't succeed to build an efficient defense mechanism. However, we found that many genes encoding enzymes involved in the biosynthesis of phenylpropanoids are up-regulated including genes encoding caffeoyl-CoA 3-O-methyltransferase and chalcone synthase (Figure 6). Also, the gene encoding PEP carboxylase was up- regulated in our experiment; this enzyme can convert 3-dehydroshikimate to p-coumarate, which is the substrate driving the production of phenylpropanoid compounds.

There were 316 up-regulated genes that shared similarity with genes encoding known proteins, while 15 genes shared similarity with unknown proteins and 49 genes were not similar to other genes (e-value $\leq 10^{-2}$). The fifty most highly induced genes are listed in Table 2. Most of the up-regulated genes with similarity to genes encoding known proteins were related to defense and disease and metabolism (Figure 4B).

Some genes encoding proteins involved in plant defense were up-regulated, but no defense pathway appeared to be completely activated at the transcript level. Genes were induced that encode β-1,3-glucanase, glutathione S-transferase, thaumatin –like protein, osmotin, disease resistance-responsive proteins-related, stress-induced protein and the pathogenesis-related protein 10 (PR-10). All are induced by salicylic acid (SA), which is produced in response to pathogen infection (Murphy et al., 2000; Narusaka et al., 1999). However, no genes encoding enzymes necessary for synthesis of SA were noted as induced. Other genes, such as those encoding polyphenol oxidase and cysteine protease inhibitor, which are induced following jasmonic acid (JA) synthesis (Thaler et al., 2001; Farmer et al., 1992), were also induced. Also, genes were enduced that encoded the enzymes 12-oxophytodienoate
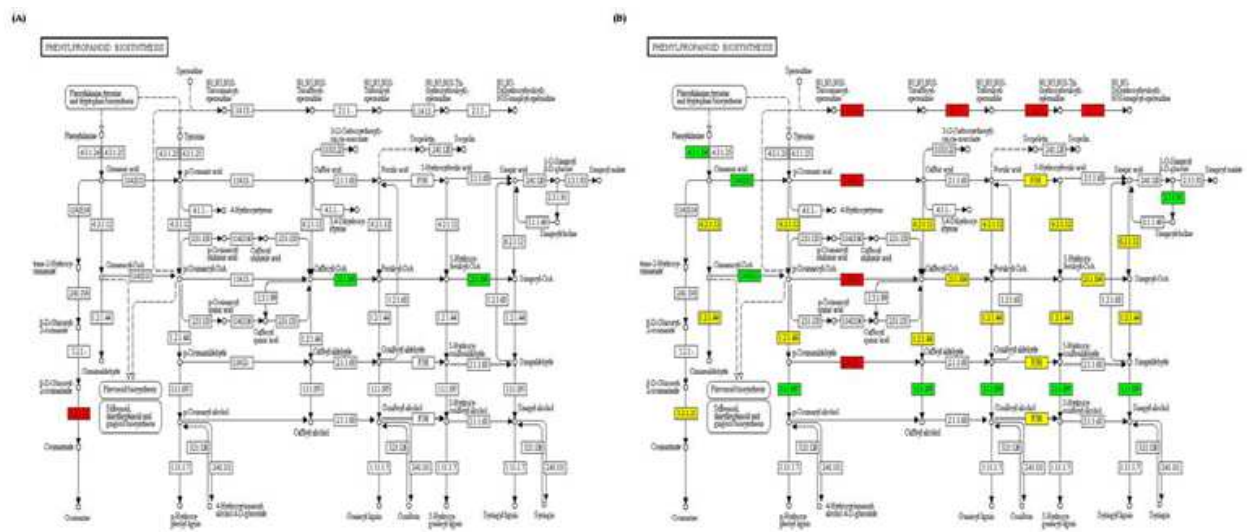
Fig. 6. Expression levels of genes encoding enzymes overlaid on the phenylpropanoid biosynthesis pathway.

reductase and allene oxide synthase (AOS) that are involved in JA synthesis. However phospholipase A2 and lipoxygenase 2 were suppressed. A gene encoding chitinase IV was up-regulated as were genes encoding chitinase proteins. These are well known digestive enzymes that break down glycosidic bonds in chitin which comprise the cell wall of fungi. They are also induced following JA synthesis (Davis et al., 2002; Rakwal et al., 2004). However, induction of all these genes is not sufficient to elicit an effective defense response against SR in this susceptible soybean cultivar, but it is apparent that the plant continues to fight the infection process by expressing defense-related genes.

One condition that contributes to the rust infection on grasses is low nitrogen levels in the soil (Doubrava and Blake, 1999; Hagan, 2005). By shutting down nitrogen metabolism, the fungus improves its ability to infect. Indeed, a decrease in nitrate reductase activity has been observed by Sadler and Scott (1974) during the first 2 days of infection of barley leaves by *Erysiphe graminis*. However, most studies report that nitrogen assimilation increases after this early stage of infection (Jenkin, 1977; Walters et al., 1984). van de Mortel et al. (2007) demonstrated that there is a biphasic change in mRNA accumulation in response to SR infection. There is an increase in levels of transcripts encoding enzymes involved in nitrogen metabolism after 24 hai, then a decrease at 10 dai. In our experiment, we also saw a decrease in expression of genes encoding enzymes involved in nitrogen metabolism including genes that encode nitrate reductase, nitrilase, glutamate synthase, glutamine synthase, and aminomethyltransferase. However, we found that transcripts encoding asparagine synthase, also involved in nitrogen metabolism, had a different expression profile at 10 dai, its expression increased. Asparagine synthase activity has been reported to increase in barley leaves infected by *E. graminis* at later stages of infection (Sadler and Scott, 1974). However, in barley other enzymes (NAD+ glutamate dehydrogenase, NADP+ glutamate dehydrogenase and glutamine synthase) involved in nitrogen assimilation were also increased. In our experiments with soybean, asparagine synthase expression could ultimately be used to produce alanine, a precursor of pyruvate which would allow the soybean leaves infected with SR to produce ATP via the TCA cycle.

Many studies on biotrophic interaction between a fungus and its host plant show that translational activity as well as ribosomal biogenesis is reduced at the early stage of

infection reflecting a major change in gene translation (Mould and Heath, 1999; Heath, 1997). Yamamoto et al. (1976) demonstrated that this reduction in protein synthesis is really specific to the early stage of infection up to 3 dai. After 3 dai, protein synthesis increases (Heinz et al., 1990). On the other hand, Polesani et al. (2008) showed at the latest stage of infection of grapevine with *Plasmopara viticola*, that genes involved in protein metabolism are predominantly repressed suggesting a repression of protein synthesis and turnover (Polesani et al., 2008). These results are in agreement with our data which show a down-regulation of protein synthesis at 10 dai reflected by the down-regulation of many genes encoding ribosomal proteins such as the large subunit of the ribosomal protein L3 and the small subunit of the ribosomal protein S17.

If we compare our study resulting from laser capture microdissected tissues to the study of van de Mortel *et al.* (2007) using whole leaves of a susceptible genotype at 7 dai, we find that out of our 380 up-regulated genes, 75 were identified in infected leaf tissue by van de Mortel et al (2007) as well, while 305 genes were unique, identified only in our experiment which focused on gene expression specifically at the infection site. All genes classified in our dataset as related to cell growth and division and intracellular traffic functional categories were unique. Eighty percent of genes we identified as members of the energy category were unique including phosphoenolpyruvate (PEP) carboxylase. About 75% of our genes included in cell structure, protein destination and transcription categories were unique to our experiment. Finally, 58% of our genes classified as involved in signal transduction were unique to our LCM isolated material. Out of 1,640 down-regulated genes identified in our experiment, 1,601 were unique as compared to the results of van de Mortel *et al.* (2007). Of the 39 genes in common with those identified by van de Mortel *et al.* (2007) were triosephosphate isomerase and transketolase involved in carbon fixation and many 50S ribosomal proteins. These results indicate that there are many different changes occurring between 7 and 10 dai in a susceptible soybean plant infected by SR. Some changes have already been seen in other fungi including other rusts but many changes seem to be specific to SR.

4. Deep sequencing assay to identify SR and soybean genes from infected plants

Since 2004, many companies have been working in the area of next generation sequencing to provide high throughput and deep sequencing technology at low cost. In 2006 Solexa developed a next generation genetic analysis system. This system has been used for multiple applications rely on sample preparation. It allows DNA sequencing, transcriptome analysis and gene regulation analysis. RNA analysis through the mRNA-Seq assay to provide transcriptome analysis has been available since October, 2008. Since then, there have been numerous manuscripts describing the utilization of mRNA-Seq application on the Solexa platform to perform transcriptome analysis firstly focusing on mammals and yeasts, and more recently on plants. However, only few analyses have been done on infected plants.

4.1 Methodology
We have performed an mRNA-Seq assay using the Solexa platform to test the potential offered by this new technology to identify more SR and soybean genes expressed under specific conditions as well as new genes from both organisms. Williams 82 soybean trifoliate leaves were collected at 240 hai and before inoculation (time-zero). Fragmented mRNA from

these samples was used as template for cDNA synthesis using a standard protocol. The Genomic DNA Sample Preparation Kit from Illumina was used to complete the cDNA library preparation.

Four picomolar of cDNA at 240 hai and eight picomolar of cDNA at time-zero were hybridized to the flow cell surface in a cluster generation station (Illumina). The libraries were sequenced as 36-mers for 240 hai and 80-mers for time-zero on the Genome Analyzer IIx (GAIIx) (Illumina). Illumina Genome Analyzer pipeline v1.4 has been used to analyze the images generated by the GAIIx, to do the base-calling and to align reads onto a reference Soybean genome. In aligning of RNA-Seq reads to the Soybean reference genome, the Soybean (*Glycine max*) v5.0 genome build was utilized (www.phytozome.net/soybean).

In relation to the Soybean v5.0 build used for read mapping, corresponding functional annotations were derived from the JGI Soybean ftp site (ftp://ftp.jgi-psf.org/pub/JGI_data/phytozome/v5.0/Gmax). Mapping of reads and derivation of transcript abundance was made possible using TASE (Hosseini et al., 2010), an open-source read-counting tool designed for Solexa datasets. TASE enables the ability to calculate how many reads mapped onto genomic transcripts thereby providing a mode of transcript expression. The resulting number of reads have been normalized by divided the number of reads for a given gene in an experiment by the total number of annotated reads in the same experiment multiplied by a constant (100,000). Executing TASE was made possible using KOG (clusters of eu**K**aryotic **O**rthologous **G**roups) homology-based annotations, Soybean build v5.0 mRNA transcript genomic start and end sites, and the Solexa dataset containing reads for all sequenced lanes. Output from TASE contained the genomic region for a given transcript (in a specific chromosome and lane), with the number of reads mapping to that given region. PAICE was used to overlay genes expressed on different metabolic pathways and color enzymes.

4.2 Results and discussion

Solexa reads were aligned to the soybean genome using Phytozome version 5.0. Three categories of reads were identified. First were reads aligning to annotated regions of the soybean genome. Second were reads aligning to regions of the soybean genome wherein no annotation is present to indicate the presence of a gene. However, many of these unannotated regions appear to containing genes according to our data. Approximately 61 percent of the total number of reads (around 15 million reads) aligned to the soybean genome and fell into the first two categories. In spite of efforts to identify the function of all protein-coding loci, there are still unannotated regions of the genome that encode mRNA regions without any functional annotation. We refer to these regions as positionally-unannotated genes. Among all reads aligning to the soybean genome, an average of 54 percent mapped to positionally-annotated genes which represent over 18,000 protein-coding loci, while the remainder of these reads mapped to positions currently unannotated in Phytozome. Sequence homology searches of the positionally-unannotated genes using several different public databases such as NCBI and InterPro revealed that 70% of the sequences share similarity with genes found in other plant organisms. Approximately 30% of sequences remained unknown.

The third category of reads contains those that did not align to the soybean genome. About 39 percent (around 10 million) of reads fall into this third category. These may represent soybean genes encoded in gaps in the present version of the soybean genome or they may be SR genes. Some of these reads could be positively identified as being SR genes, however,

the sequence and assembly of the genome of SR is not available. Some of these reads had high identity with genes of other fungi. The majority of these reads did not have high homology to other fungal genes nor the limited number of known SR genes. We presume that many of these represent newly discovered SR genes.

4.2.1 Soybean gene expression profile

To characterize the differential expression of soybean genes at 10 dai, we analyzed the tag count for a specific gene at 10 dai sample versus the tag count at time-zero which give us a fold-change. Forty-three percent of our positionally-annotated genes were down-regulated at 10dai while 31% were up-regulated. Twenty-six percent seemed to maintain the same expression level at 10 dai compared to time-zero. Figure 7 shows in which functional category down- and up-regulated genes are involved.
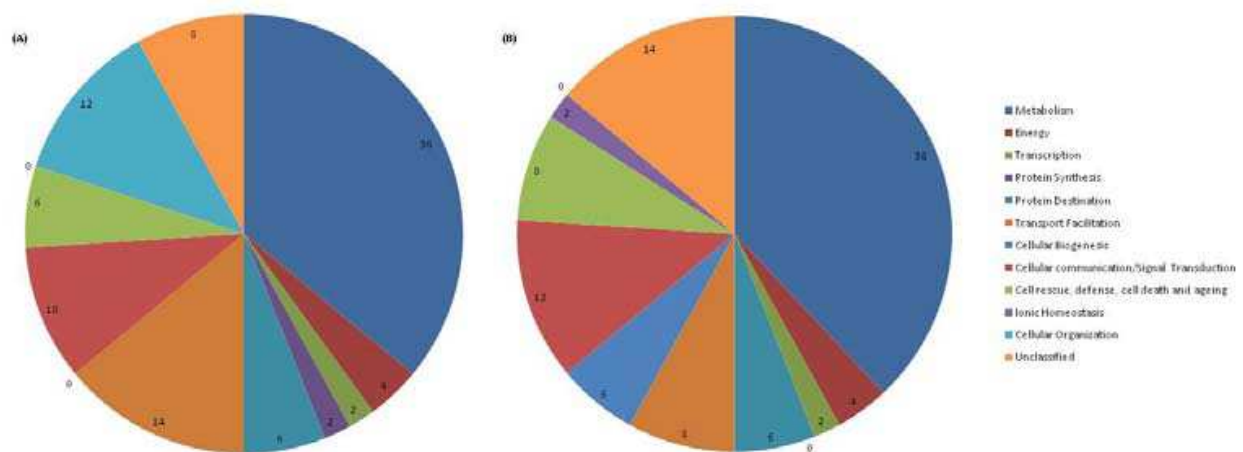
Fig. 7. Functional categorization of (A) down- and (B) up-regulated genes expressed in soybean Williams 82 10 dai with SR compare to time-zero.

Compared to microarrays, deep sequencing technology allowed the identification of 17,363 more genes and changes in transcripts of genes encoding proteins of seventy-one more metabolic pathways. General trends seen from the microarray results were still observed using deep sequencing technology which means that more genes were down-regulated. They were mostly involved in carbohydrate metabolism and protein synthesis but they were also involved in nitrogen metabolism and carbon fixation. Fewer genes were up-regulated and they were mostly involved in general defense mechanisms. In the down-regulated pentose phosphate pathway, there more genes found expressed using deep sequencing (Fig.5B) than microarrays (Fig.5A). The same observation can be done for the up-regulated phenylpropanoid biosynthesis pathway (Fig.6) where more genes encoding enzymes were found expressed using deep sequencing (Fig.6B) than microarray analysis (Fig.6A). Finding more genes allowed confirming or refuting the regulation of many metabolic pathways observed with our microarray results and also allowed linking between pathways.

While microarrays showed down-regulation of many genes encoding proteins involved in protein synthesis, our deep sequencing results showed that numerous genes encoding enzymes involved in almost all amino acid metabolic pathways were down-regulated as well. Most of the encoding genes involved in glycine, serine and threonine metabolism were down-regulated but the serine-glyoxylate transaminase, responsible for the conversion of

glycine to serine to hydroxyl-pyruvate leading to the glyoxylate/dicarboxylate metabolism, was clearly up-regulated. Since the glyoxylate/dicarboxylate metabolism is one of the only pathways in carbohydrate metabolism found up-regulated, the up-regutation of this specific gene make sense. Glyoxylate may be used by malate synthase to produce (S)-malate and subsequently malate dehydrogenase would produce oxaloacetate, which could enter the pathway for pyruvate metabolism or vice versa. This latest observation supports the belief that pyruvate metabolism is truly up-regulated. Since glyoxytate can be produce by purine metabolism, genes encoding enzymes responsible for its production have been found expressed and up-regulated which refute microarray results indicating otherwise. Successful development of many rust and mildew infections requires an adequate and uninterrupted supply of nutrients from the host. Purines are nutrients needed for many metabolic events in addition to nucleic acid synthesis in eukaryote organisms. With pyrimidines, they are essential constituents of cytokinines (D'Agostino and Kieber, 1998). As cyclic monophosphates and as ADP-ribose they are also involved in signal transduction (Scheel, 1998). Moreover, they are involved in the synthesis of secondary products (Suzuki and Takahashi, 1977). An obligate biotroph which is unable to synthesize purines *de novo*, must obtain these nutrients from its host. Purines may be derived from the breakdown of host nucleic acids and some enzymes involved in purine salvage are present in both host and pathogen. In barley infected with powdery mildew (Butters et al., 1985), appressoria metabolize adenine and adenosine and converted these compounds mostly into inosine. Guanosine metabolism was more limited and hypoxanthine was neither absorbed nor metabolized. In a healthy plant, adenosine was converted into other purines, but inosine was not the major metabolite. In soybean infected with soybean rust, changes in purines metabolism were also observed. Genes responsible for guanine and xanthine production seemed to be up-regulated compare to those responsible for hypoxanthine and adenine production.

Even if microarrays showed that genes encoding enzymes involved in terpenoid and steroid biosynthesis pathways were down-regulated, it did not showed any expression of genes encoding proteins involved in brassinosteroid biosynthesis. In our deep sequencing experiment, many genes encoding enzymes involved in this pathway were found as down-regulated. Brassinosteroids (BRs) are a class of plant polyhydroxysteroids that are ubiquitously distributed in the plant kingdom. BRs are involved in many different cellular responses such as cell elongation, pollen tube growth, xylem differentiation, leaf epinasty, and root inhibition (Clouse and Sasse, 1998; Mandava, 1988). Their role in plant resistance to diverse environmental stresses has been confirmed in many studies (Ikekawa and Zhao, 1991, Kamuro and Takatsuto, 1991). The potential of BRs to enhance plant resistance specifically against fungal pathogen infection has been investigated as successful in many studies (Khripach et al., 1999, 2000). However, BRs have also been found as stimulant for fungal growth and disease progression (Krishna, 2003) which mean that protective or deprotective type of BRs activity depend on the concentration of BRs, where it is expressed and is connected with the different stimulating points of either the plant or the pathogen (Korableva et al., 2002). Since most of the gene family involved in BRs biosynthesis included up and down-regulated genes, it is hard from our experiment to know if this pathway was up or down-regulated. But the interest stills the same about the involvement of BRs in plant defense.

Genes involved in transport facilitation were strongly down-regulated. These genes mostly encoded aquaporins. In many cases infection of plants by pathogens can lead to water stress.

In plants compatible to *Phytphthora* spp. there is a decline in stomatal conductance upon infection, which can cause water stress (Manter et al., 2007). Aquaporin encoding genes, responsible for water transport, occur as multiple isoforms. For example, *Arabidopsis thaliana* encodes 35 aquaporin homologues and maize encodes at least 31 aquaporin homologues (Luu and Maurel, 2005; Chaumont et al., 2001). This may explain the high number of aquaporin encoding genes found in our fifty most down-regulated genes. On the other hand, down-regulation of genes encoding aquaporins in soybean infected by soybean rust is similar to what has been reported in esca-infected grapevine where some aquaporin encoding genes were repressed at late stages of the infection (Letousey et al., 2010).

Deep sequencing allowed the analysis of expression of genes encoding proteins associated with salicylic acid (SA) biosynthesis, which is involved in defense. Genes encoding chorismate mutase, anthranilate synthase, isochorismate synthase as well as phenylalanine ammonia-lyase were identified and expression analyzed. Some of these genes were not present on the soybean array while some other were not found expressed using microarray. Genes involved in other biosynthetic pathways closely related to defense were also up-regulated such as genes encoding enzymes involved in jasmonic acid and ethylene production but in less abundance.

Another set of up-regulated genes encoded enzymes involved in glycan metabolisms. In eukaryotic cells, both soluble and membrane proteins that enter the endoplasmic reticulum (ER) system may undergo a posttranslational modification called *N*-glycosylation. *N*-glycosylation occurs in two phases, namely, core glycosylation in the ER and glycan maturation in the Golgi apparatus (Kukuruzinska and Lennon, 1998; Helenius and Aebi, 2001). The biological functions of *N*-glycan modifications in the Golgi apparatus are well established in humans, insect and yeast (Sarkar et al., 2006; Bates et al., 2006). In plants, the first *Arabidopsis thaliana* mutant lacking complex *N*-glycans was reported in 1993 (von Schaewen et al., 1993). Plants with altered *N*-glycan modification pathways that are devoid of potentially immunogenic complex *N*-glycans are mostly used for the production of pharmaceutical proteins (Strasser et al., 2008; Koprivova et al., 2004) and could serve as potential food crops with reduced allergenicity. Until recently, however, complex plant *N*-glycans have not been associated with essential biological functions in their host plants due to lack of obvious phenotypes of mutant plants defective in complex *N*-glycan biosynthesis. Kang *et al.* (2008) recently reported that mutants defective in complex *N*-glycans show enhanced salt sensitivity, establishing that complex *N*-glycans are indispensable for certain biological functions. However, there is still no report on the involvement of N-glycans in response to biotic stresses. Our study showed that genes encoding enzymes involved in N-glycan biosynthesis as well as genes encoding enzymes involved in high-mannose type N-glycan biosynthesis were mostly up-regulated. Additional investigations on their role in plant-pathogen interaction need to be done.

Genes involved in intracellular communication/signal transduction comprised 12% of the most up-regulated genes. These mostly included genes encoding serine/threonine protein kinases. The network of protein serine/threonine kinases in plant cells appears to act as a "central processor unit", accepting input information from receptors that sense environmental conditions, phytohormones, and other external factors, and converting it into appropriate outputs such as changes in metabolism, gene expression, and cell growth and division (Hardie, 1999).Since these genes are involved in many biological processes, their presence in the most up-regulated genes was expected.

4.2.2 New soybean genes

The second category of reads are reads that align to the soybean genome on a position not yet annotated for presence of a gene or not yet annotated for functionality. This category includes about 5,500,000 reads. These reads were assembled into to get contigs using Velvet (Zerbino and Birney, 2008). About 12,000 contigs were built ranging from 30 to 801 nucleotides. Open reading frames were found for each of these contigs and used to find similarity against NCBI proteins database and Interpro database for conserved domains. Table 3 shows a subset of contigs not yet annotated but sharing similarity with gene encoding proteins, gene encoding conserved domains and/or microRNA (miRNA) from these different databases. Even if only few new genes shared similarity to genes directly related to defense, this additional information will be useful to complete annotation of the soybean genome sequence.

Chromo Source	Contig Length (bp)	Chromo Location	Protein Description (Evalue)	Conserved Domain	MiRNA (Evalue)
Gm07	166	9210964-9310713	*G. max* unknown (-07)	Signal peptide, Transmembrane regions	*Physcomitrella patens* miR414 (-05)
Gm11	249	18746483-18786222	*Glycine max* proline-rich cell wall protein 3 (-77)	NA	NA
Gm14	327	6192322-6213926	NA	NA	*Populus trichocarpa-* miR530b (-04)
Gm14	201	4037946-4050174	*Vitis vinifera Vitis vinifera* Top of Form hypothetical protein (-05)	Copper transport protein ATOX1-related	NA
Gm15	153	3369602-3374061	*Arabidopsis thaliana* Top of Form HIS4; DNA binding (-84)	Histone H4	NA
Gm17	185	24487406-24721204	*G. max* SRC1 (E-49)	Signal peptide, Transmembrane regions	NA

Table 3. Contigs displaying similarity to proteins, conserved domains and miRNA in the nonredundant protein NCBI, InterPro and miRNA databases. NA for none applicable.

Since their discovery in 1993, miRNAs have been largely identified in eukaryotic organisms (Lee et al. 1993). They are post-transcriptional regulators playing multiple roles in negative regulation (transcript degradation and sequestering, translational suppression) and possible

involvement in positive regulation (transcriptional and translational activation). By affecting gene regulation, miRNAs are likely to be involved in most biologic processes (Cuellar and McManus, 2005; Wilfred et al., 2007). In plants, miRNAs function to control tissue (leaf, root, stem, and flower) differentiation and development, phase switching from vegetative growth to reproductive growth, signal transduction, and the response to biotic and abiotic stress (e.g., salinity, drought, and pathogens) (Chen 2005; Zhang et al. 2006). There are 229 miRNA identified in soybean while there is about 1441 miRNAs identify in *A. thaliana*, 2641 in rice, 2780 in *Populus trichocarpa*, and 395 in *Medicago truncatula* (Zhang et al., 2010). Their identification is still important since they may play a role in plant defense.

There was also one contig sharing similarity to a gene encoding cold-stress protein src1 from *G. max* itself. As it name tell us, the expression of this gene is activated early in response to temperature stress (Takahashi and Shimosaka, 1997). However, an increase in its transcript abundance had also been observed following drought, wounding and infection with soybean mosaic virus. Theis evidence showed that this gene may be involved in plant defense against pathogens.

The expression of genes encoded by contigs aligning to the soybean genome on a position not yet associated with functionality and by contigs corresponding to potential new genes without similarity to known sequences, will be compared with that in *A. thaliana* to try to find information about their role in plant development and maybe plant defense.

4.2.3 Soybean rust gene expression

The third category of reads are reads not aligning to the soybean genome included about 10,000,000 reads. These reads were assembled into contigs using Velvet. About 33,000 contigs have been built ranging from 50 to 1,991 nucleotides. An automated homology search against NCBI database found that only about seven percent of the contigs shared similarity to annotated genes. This result reflected the lack of information concerning filamentous fungi in general. Figure 8 shows with which functional category these genes have been associated.
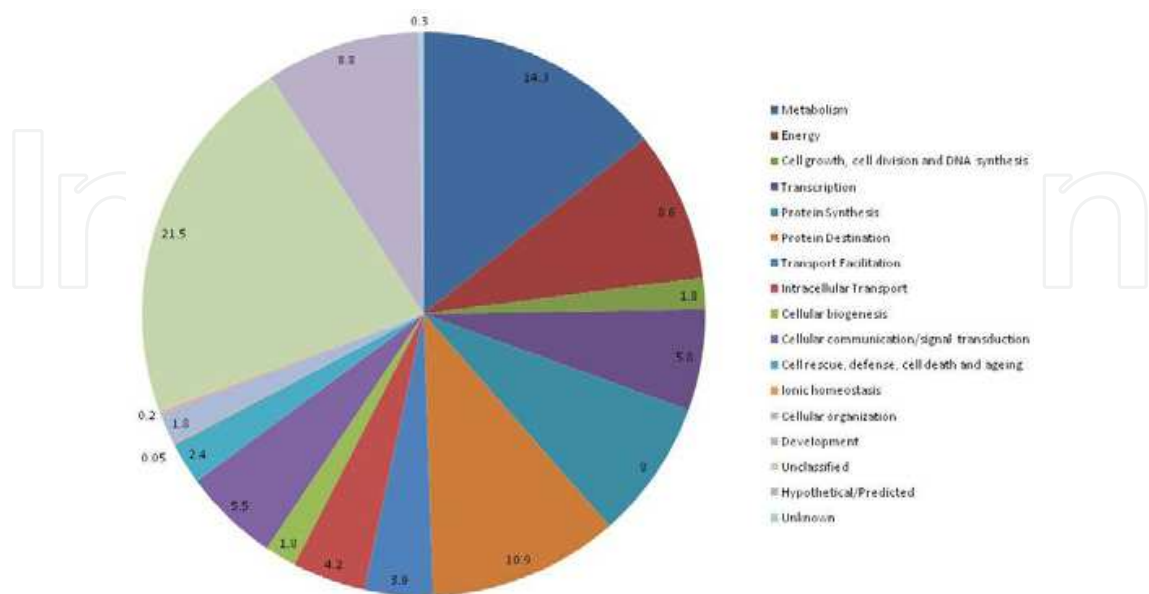

Fig. 8. Functional categorization of soybean rust genes expressed in soybean Williams 82 10 dai.

Metabolism and protein destination were the two functional categories where most of the annotated SR genes were included.

In metabolism category, the amino acid metabolism and the carbohydrate metabolism were the most populated with genes. Many genes encoding enzymes involved in cysteine and methionine metabolism have been found including genes encoding S-adenosylmethionine decarboxylase, adenosylhomocysteinase and 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase. Mutation of *Fusarium graminearum* cystathionine beta-lyase gene and methionine synthase gene involved in cysteine and methionine metabolism have been reported to reduce virulence of the fungus indicating that methionine synthesis is critical for plant infection (Seong et al., 2005). In *Magnaporthe grisea*, a methionine auxotrophic mutant was also reduced in virulence (Balhadere et al., 1999) and mutant defective for methionine synthase gene expression of *Cryptococcus neoformans* was nonpathogenic (Pascon et al., 2004). Auxotrophic mutants of other fungal pathogens defective in other amino acid metabolisms, such as histidine synthesis in *M. grisea* (Sweigard et al., 1998) and arginine synthesis in *Fusarium oxysporum* (Namiki et al., 2001), have also been reported to be reduced in virulence.

Many genes encoding enzymes involved in carbohydrate metabolism have been identified. Some genes were encoding enzymes involved in glyoxylate metabolism such as malate dehydrogenase and malate synthase. Other genes were encoding enzymes involved in citrate cycle such as aconitate hydratase, succinate dehydrogenase, citrate synthase and isocitrate dehydrogenase. Presence of these enzymes as well as other involved in citrate cycle and glyoxylate metabolism in rust spores has been reported by Caltrider et al. (1963), Frear and Johnson (1961), and Staples and Stahmann (1964). This suggested that the TCA cycle and its glyoxylate shunt are present in urediniospores and are potentially very active. Small amounts of substrates which penetrate urediniospores membranes are rapidly converted via these pathways to the numerous products.

In protein destination, we have found genes encoding proteins such as ubiquitin-conjugating enzyme, ubiquitin-activating enzyme, proteasomes and different proteases. This kind of enzymes has been often found associated with the fungus germination and appressorium formation steps. Liu and Kolattukudy (1998) found an ubiquitin-conjugating enzyme induced by hard surface contact of *Colletotrichum gloeosporioides* conidia. Kim et al., (2004) were studying the proteome of *Magnaporthe grisea* and found that 20S proteasomes were present during appressorium formation. Fungal genes encoding proteases such as aspartyl proteases and serine proteases mostly belonging to the subtilisin class have been discovered to be expressed during infection (Mendgen and Deising, 1993; Carlile et al., 2000). Their role into pathogenicity is still circumstantial but there is some evidences suggesting a direct action of fungal proteases on host plant proteins from plasma membrane (Tseng and Mount,1974) or cell wall (Movahedi and Heale, 1990) for example, by degradation of plant cell wall proteins (Carlile et al., 2000; Dow et al., 1998;). Fungal proteases have been also found involved in the adhesion of fungal pathogens to host cells (Borg von Zepelin et al., 1999). Indirect evidence for a role of proteases in plant disease is that plants frequently react to microbial infections by the accumulation of fungal protease inhibitors (Chen et al., 1999; Vernekar et al., 1999). However, there is no mention in the literature about expression of fungal proteases late in the infection process but they also may be involved in degradation of cell wall proteins to allow expulsion of mature spores from the host in combination to turgor pressure.

For the remaining 93% of the contigs, open reading frames have been found and used to manually find similarity against NCBI proteins database, Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) proteins database specific for phytopathogenic fungi and oomycetes and Interpro database for conserved domains. This additional analysis allow us to identify more genes sharing similarity to genes encoding proteins involved in many biological processes such as transcriptional control (LIM class homeodomain transcription factor, Lhx6/8 subclass from *Branchiostoma florida*), translation (translation elongation factor from *Cryptococcus neoformans* var. *neoformans*), lipids, fatty acid and isoprenoid biosynthesis (acetyl-CoA acetyl transferase and cyclopropane-fatty-acyl-phospholipid synthase from *Laccaria bicolor*, fatty acid elongase from *Saccharomyces cerevisiae*), proteolysis and protein folding (dipeptidyl peptidase III from *Neosartorya fischeri*, FK506-binding protein 3 from *Pyrenophora tritici-repentis*, Hsp90 co-chaperone Cdc37 from *Paracoccidioides brasiliensis*) and glycolysis (mitochondrial pyruvate dehydrogenase E1 component beta subunit from *L. bicolor*).

Genes sharing similarity to genes encoding protein involved in defense against oxidative stresses such as a catalase from *Botryotinia fuckeliana* and *Taiwanofungus camphorates* and a peroxidase from *Talaromyces stipitatus* and *Gibberella zeae* have also been found. Plants defend themselves against pathogen attack by invoking a myriad of mechanisms, including the hypersensitive response, the production of antimicrobial metabolites, cell-wall fortification and the papilla response. One of the most rapid responses to pathogen attack is the production of active oxygen species (AOS) at sites of invasion, named the oxidative burst (Zhang et al., 2004).On the other hand, very little is known about the effect of AOS and the response to AOS from the pathogen perspective. If AOS do have roles in plant defence then the evolution of antioxidant detoxifying systems may arm a pathogen with a major selective advantage in possible suppression of host defenses. But what may be the function of this detoxifying system at late stage of the infection still to be understood.

During infection process, fungi face the challenges of recognizing susceptible hosts, overcoming physical and chemical barriers to invade plant tissues, withstanding host defense mechanisms, extracting nutrients for proliferation, and eventually disseminating to a new host. Morphological transitions during the infection process are an integral aspect of fungal phytopathogenesis. In this regard, the virulence arsenal of a fungal phytopathogen may include the ability to exhibit reversible switching between specialized morphological types of cells, the production of infection structures such as appressoria or haustoria, and the ability to produce spores as agents of dispersal. These morphological aspects of virulence are tightly regulated by environmental signals and signaling pathways that operate throughout infection. Fungal cells employ sophisticated signal transduction programs to sense and respond to specific environmental cues that drive changes in cell morphology and physiology. Signaling mechanisms that regulate fungal proliferation and differentiation include the highly conserved MAP kinase and cAMP (cyclic adenosine monophosphate) signaling cascades (Lee et al., 2002b). Probably part of the signaling pathway in SR, we also found an additional gene sharing high similarity to gene encoding a protein involved in intracellular communication and signal transduction, the serine/threonine protein phosphatase PP1 from *Coprinopsis cinerea okayama*.

Genes sharing similarity to genes encoding proteins involved in the biogenesis of fungus cell wall have also been identified including genes encoding chitinase from *Puccinia triticina* and chitin deacetylase from *Alternaria brassicicola*.

In addition, couple more genes encoding proteins containing a signal peptide have been pointed out.

5. Conclusion

In this study, we focused on a susceptible interaction between one US commercial soybean cultivar and one US SR isolate. This specific interaction was chosen so that we could obtain enough material during the time-course to be able to study gene expression specifically in specific infection sites. In spite of obtaining a limited number of unisequences from the cDNA library sequencing, this experiment allowed us to identify a number of novel fungal mRNAs expressed in uredinia that are in some way associated with infection and/or proliferation.

Some of the fungal genes from our cDNA library sequencing in addition to fungal genes from the deep sequencing assay may be further examined through gene silencing in soybean infected with *P. pachyrhizi*. Genes that contain signal peptides represent viable targets for gene silencing as well as genes encoding proteins involved in defense against oxidative stresses and biogenesis of cell wall to delay or even stop the infection process.

Some of the soybean genes found expressed by our microarray assay represent interesting candidate genes for over-expression to control SR infection. For example, the reduction in nitrogen metabolism and protein synthesis that occurs in the plant at the end of the infection process may be an important trigger for sporulation of the fungus. Zuk et al. (2005) found that a repression of 14-3-3 proteins, well known for their interaction with other proteins, affect nitrogen fixation by regulating nitrate reductase (NR). Since NR is a key enzyme in nitrogen assimilation, they found that repression of these 14-3-3 proteins also boosts protein content. It may be possible to use 14-3-3 proteins to up-regulate instead of down-regulate nitrogen metabolism and protein synthesis at 10 dai with ASR and perhaps stop the fungus sporulation and its spread into the environment. Another example may be the over-expression of genes involved in purine metabolism. This may change the metabolite balance in favor of the plant and inhibit fungal sporulation.

Overall, our experiment provides new information about the infection process both from the pathogen side and from the plant side. It gives us a better understanding of gene expression in the fungus and the plant during the life cycle and this may help us to identify approaches to broaden the resistance of soybean to SR. It provides insight into the needs of the pathogen and what genes are required and not needed for the pathogen to sporulate. Furthermore, our results show that the LCM procedure is a valid and powerful tool to collect specific infected portions of the plant for further examination. Thus, we can study gene expression patterns in a very specific infection structures, such as in the uredinium of SR, find genes essential to fungus development, and use those genes to try to build resistance into the plant. Moreover, our results using deep sequencing technology shows the performance of this quite new technology to increase our knowledge in a time effective manner. Finally, our study expands our knowledge about the development of this new exotic pathogen that threatens soybean production.

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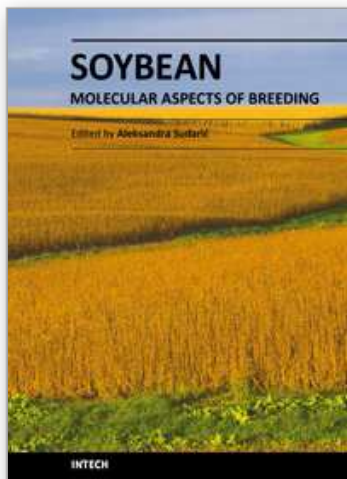
Soybean - Molecular Aspects of Breeding

Edited by Dr. Aleksandra Sudaric

ISBN 978-953-307-240-1

Hard cover, 514 pages

Publisher InTech

Published online 11, April, 2011

Published in print edition April, 2011

The book Soybean: Molecular Aspects of Breeding focuses on recent progress in our understanding of the genetics and molecular biology of soybean and provides a broad review of the subject, from genome diversity to transformation and integration of desired genes using current technologies. This book is divided into four parts (Molecular Biology and Biotechnology, Breeding for Abiotic Stress, Breeding for Biotic Stress, Recent Technology) and contains 22 chapters.

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