Transcriptional profiling of potential regulatory factors modulating defense mechanisms in soybean during *Phytophthora sojae* infection.

LaChelle Waller

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Brett M. Tyler, Chair
M.A. Saghai Maroof
Ina Hoeschele
John McDowell

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Abstract:

Transcriptional profiling of potential regulatory factors modulating defense mechanisms in soybean during *Phytophthora sojae* infection

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Brett Tyler, Chair

Quantitative resistance is controlled by multiple genes and has been shown to be a durable form of resistance to pathogens affecting cultivated crops including soybeans (*Glycine max* L. Merr). Root rot of soybean caused by *Phytophthora sojae* ranks among the most damaging soybean diseases. Quantitative resistance has proven durable in soybean against *P. sojae,* however the molecular mechanisms underlying this form of resistance are still unknown. The objective of this project is to gain insight into molecular basis of quantitative resistance in the soybean-*P. sojae* pathosystem. The approach was to use global transcriptional profiling based on microarray technology to identify genes that were differentially expressed in four cultivars of soybeans with varying levels of quantitative resistance at different time points during infection by *P. sojae*. Our results provide a better understanding of the potential regulatory factors that may contribute to quantitative resistance during early hours of *P. sojae* infection.

To my mother and father,

To Destiny Waller

In memory of my Grandmother Madeline Waller

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This project truly depicts the true example of transdisciplinary team science research.

List of Abbreviations

QTL	Quantitative trait loci
MLG	Mapping linkage group
SAR	Systemic Acquired Resistance
MAMPs	Microbe-associated molecular patterns
PAMPs	Pathogen-associated molecular patterns
PTI	PAMP - triggered immunity
ETI	Effector - triggered immunity
PRR	Pattern recognition receptor
LRR	Leucine rich repeat
FLS	Flagellin Sensitive 2
QR	Quantitative resistance
HR	Hypersensitive response
ROI	Reactive oxygen Intermediate
BAC	Bacteria Artificial chromosone
BZIP	basic Leucine Zipper
EST	Expressed sequence tags
CERK	CHITIN ELICITOR RECEPTOR KINASE 1
DRE	DEHYDRATION-RESPIONSIVENESS ELEMENT
ETH	Ethylene
JA	Jasmonic acid
SA	Salicylic acid
NO	Nitric oxide
RFLP	Restriction Fragment Length Polymorphism
FDR	False discovery rate
ROS	Reactive oxygen species
TFs	Transcription factors

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Chapter 1

Introduction

Rationale

Quantitative resistance is a durable means of disease control in soybean (*Glycine max* L.Merr). However, it provides only partial protection and is difficult to breed for. Currently soybean breeders rely heavily on R-gene-mediated resistance against the root rot pathogen *Phytophthora sojae (P. sojae)* to limit disease in soybean cultivars. Due to changes in the *P. sojae* populations that have reduced the effectiveness of R gene mediated resistance, soybean breeders are placing increased emphasis on quantitative resistance. Recent research has shown that quantitative resistance is controlled by multiple genes in soybean and is not easily overcome by changes in the *P. sojae* population.

Research Objectives

The purpose of this study is to identify regulatory factors present during early time points of infection that may control quantitative resistance in soybean cultivars. Analyzing microarray data generated from experiments done on soybean cultivars with varying degrees of resistance to *P. sojae* will help determine the molecular basis underlying quantitative resistance against *P. sojae,* which will ultimately aid in the development of *P. sojae* management strategies. The goal of this project will be addressed by three specific aims:

Aim 1: To identify genes differentially expressed over time during infection in four soybean cultivars with varying resistance to *P. sojae*.

Aim 2: To identify regulatory genes including transcription factors in these cultivars that are specifically induced during early infection.

Aim 3: To identify regulatory genes expressed in soybean cultivars 1 day post inoculation (1 dpi) and 2 days post inoculation (2 dpi) that may be associated with known resistance QTLs.

Description of dissertation

This dissertation focuses on regulatory factors that may contribute to quantitative resistance in soybean cultivars. The final chapter of my dissertation describes an outreach program and its contribution to engaging students in transdisciplinary research. This dissertation is organized into 6 chapters.

Chapter 1 describes the motivation for the dissertation and the importance of identifying regulatory factors present during early time point infection that may contribute to quantitative resistance in soybean cultivars. The specific aims executed in this research are also documented in this chapter.

Chapter 2 presents a literature review of current knowledge regarding host-microbe interactions, the molecular basis of plant innate immunity, the regulation of plant disease, the soybean - *P. sojae* pathosystem, and finally microarray technology.

Chapter 3 presents the Materials and Methods used for the work.

Chapter 4 presents the data and results obtained for specific aim 1 followed by discussion.

Chapter 5 presents the data and results obtained for specific aims 2 and 3 followed by discussion for both aims.

Chapter 6 describes the outreach component to the dissertation. It describes the development of a community-based team science education and outreach program.

Chapter 2

Review of Literature

2.1 Plant Defense Against Infection

Plants are continually exposed to diverse microbes and defend themselves successfully against most potential pathogens. The ability of a plant to defend itself against pathogen infection is controlled by genetic factors at both the species and cultivar level (da Cunha et al., 2006). In many plant-pathogen interactions the outcome of infection is determined by the interactions between disease resistance genes (*R* genes) in plants and avirulence (*Avr*) genes in pathogens (Jones and Dangl, 2006). This type of resistance is known as cultivar resistance because only specific genetic strains of a plant species have acquired resistance to a particular pathogen (Jones and Dangl, 2006). For a particular *R* gene to be effective, the presence of a specific avirulence gene is required within the pathogen (Jones and Dangl, 2006). The hypersensitive response (HR) is an inducible plant response that is an important component of disease resistance and is associated with localized cell death (da Cunha et al., 2006). The cells contained near the infected region following HR experience a rapid death (Pinzon et al., 2009) leading to a broad spectrum and long lasting plant response known as systemic acquired resistance (SAR).

Resistance responses (leading to an incompatible interaction) allow the plant to limit pathogen growth by triggering temporary local defense measures upon recognition of avirulent pathogens (Maleck et al., 2000). In contrast, during a compatible interaction that results in plant disease, the plant does not rapidly recognize the pathogen nor mount an effective defense (Maleck et al., 2000).

In addition to *R* gene-mediated defenses, plants also exhibit basal defenses (Pinzon et al., 2009). Basal plant defenses are responsible for the ability of a plant to fend off microbes that are not adapted to that plant species, i.e. for non-host resistance. Against pathogens adapted to the host species however, these defenses are generally ineffective and weak (da Cunha et al., 2006). Basal defense responses, albeit ineffectual, may be observed when the pathogen proliferates and disease has taken place (da Cunha et al., 2006). When basal defenses are observed during

incompatible interactions they have been adequate to prevent disease by eliminating the growth of the pathogen (da Cunha et al., 2006).

Plant hormones are essential in preparing the plant to respond to potential stresses (Kazan and Manners, 2009). Plant hormones with well established roles in plant defense include salicylic acid (SA), jasmonic acid (JA) and ethylene. Auxins and abscisic acid also may significantly modulate plant defenses (Kazan and Manners, 2009). Additional plant hormones associated with plant defense and microbial pathogenesis include brassinosteroids and gibberellins (Kazan and Manners, 2009).

2.2 Molecular Basis of Plant Innate Immunity

Plants like animals, mount an innate immune response against pathogens. In plants two branches of innate immunity can be identified. The first branch results from the ability to discriminate between self and nonself (Nürnberger et al., 2004). Plants mount an inducible response to pathogens when they recognize non-self molecules such as microbial-associated molecular patterns (MAMPs; also referred to as Pathogen Associated Patterns (PAMPs) in the case of pathogens). MAMPs are molecules that are conserved and unique to broad groups of microbes (Robert A. Ingle, 2006; Vance et al., 2009). MAMPs are perceived in plants via pattern recognition receptors (PRRs) that are usually located at the plant cell surface. Recognition by PRRs leads to activation of downstream signaling which eventually results in activation of MAMP (PAMP) induced immunity (PTI) (Haitao et al., 2009). As an example, bacterial flagellin induces PTI in *Arabidopsis*, on binding to the receptor kinase FLS2 (reviewed by (Haitao et al., 2009).

Other MAMPs include cell wall glucans, tranglutaminase, and secreted elicitin lipid transfer proteins found in some oomycetes (reviewed in (Nürnberger et al., 2004; Qutob et al., 2006b). Recently, fungal chitin has been shown to interact with the Chitin Elicitor Receptor Kinase 1 (CERK1) in *Arabidopsis* (Miya et al., 2007; Wan et al., 2008). PTI is described variously as basic, basal or general resistance.

A second level of innate immunity, termed effector-triggered immunity (ETI) is observed in plants. Also known as *R*-gene-mediated resistance, or specific resistance, ETI represents a more

amplified, accelerated form of defense than PTI. ETI is initiated through plant cultivar-specific recognition of microbial effectors. Specific recognition is generally mediated via R gene products carrying leucine-rich repeats (LRRs), and is typically distinguished from PTI by elicitation of hypersensitive response (HR)-associated localized program cell death. Even though the recognition mechanisms and outcomes of PTI and ETI are different, the intermediate signaling pathways overlap (Thorsten Nürnberger, 2004).

Quantitative resistance (QR) (also termed partial resistance, horizontal resistance, multigenic resistance, rate-limiting resistance, general resistance, or field resistance) (http://www.apsnet.org/online/feature/PathPopGenetics/dorrance.html) is determined by multiple genes and unlike specific resistance, does not eliminate disease, but rather reduces disease severity by slowing disease progress (Schmitthenner, 1985). For resistance to be considered durable in a cultivar it must be effective throughout widespread cultivation in a disease favoring environment (Wang et al., 1994). In the *P. sojae*-soybean pathosystem, QR is believed to have many components which limit the infection process. These include incubation period, latent period, spore production, and infection frequency (Parlevliet, 1979; Griffiths and Jones, 1987; Rashid, 1991; Waliyar et al., 1994; Colon et al., 1995; Xue and Davidson, 1998; Dorrance et al., 2001; Mideros et al., 2007). Together these components result in reduced pathogen colonization and lesion size in soybean (Buzzell and Anderson, 1982; Tooley and Grau, 1982, 1984; Walker and Schmitthenner, 1984; Stmartin et al., 1994; Mideros et al., 2007). There are examples in which QR is controlled by a single gene of major effect, for example, the barley mlo gene (Büschges et al., 1997). PTI has been suggested to underlie non-host resistance; PTI confers robust protection against pathogenic invaders because it is durable and highly effective (Chisholm et al., 2006). Since non-host or basic resistance and quantitative resistance are both effective against all members of a pathogen species, it is possible that similar molecular mechanisms underlie these two types of resistance. The effectiveness of resistance genes can be maintained by combining partial resistance with single gene resistance to aid in the reduction and the severity of root rot and decrease the rate disease development (Burnham et al., 2003a). Partial resistance does not apply as much selection pressure on the pathogen population as specific resistance, therefore it should be more durable (Dorrance et al., 2003b; Jia and Kurle, 2008). Partial resistance is also a valuable complement to major gene resistance because it is a highly

heritable quantitative trait and even during the absence of *P. sojae* there is not a negative effect on soybean yield (Dorrance et al., 2003b; Jia and Kurle, 2008).

A recent study by Qutob and associates also suggests that basal resistance is triggered by differential recognition of PAMPs expressed during infection by *P. sojae* and it may overlap with partial resistance (Qutob et al., 2006a).

QR may correlate with the mechanisms involved in PTI, although this has not been demonstrated for a field crop (Bent and Mackey, 2007). Identifying additional sources of partial resistance and incorporation of this resistance into commercial cultivars will be essential for effective disease management as the number of *P. sojae* races increases in soybean production areas and the complexity of the virulence pathotypes of *P. sojae* continues to increase (Ranathunge et al., 2008).

2.3 Regulation of Gene Expression During Plant Disease

Regulation of gene expression in plants is important for a variety of processes including defense responses to pathogens (Kuhlemeier et al., 1987). Gene expression can be modulated from the transcription step to the post-translational modification step. Transcription is regulated by numerous transcription factors (TFs) that mediate the effects of extracellular and intercellular signals (Yanagisawa, 1998) and regulate many biochemical and physiological processes. Several transcription factors are implicated in plant defense. These include members of the following families: Ethylene Response Factor (ERF), APETALA2 (AP2)/ethylene-responsive-element-binding protein (EREBP), Homeodomain, basic Leucine Zipper (bZIP), MYB, WRKY, and other zinc finger factors. All these have been observed to increase in expression in response to pathogen challenge (Singh et al., 2002).

The ERF family has been studied at great length (Xu et al., 2008). ERF transcription factors have been identified in plants such as tobacco, rice, pepper, soybean and *Arabidopsis* (Magnani et al., 2004). In *Arabidopsis* there are estimated to be 124 ERF proteins (Riechmann et al., 2000). The ERF proteins are a subfamily of the APETALA2 (AP2)/ethylene-responsive-element-binding protein (EREBP) family which is unique to plants (Singh et al., 2002). The ERF domain is made up of a conserved 58-59 amino acid domain that binds two similar cis-elements: the GCC box,

and the C-repeat (CRT)/dehydration-responsiveness element (DRE) motif (Singh et al., 2002). Although some ERF's repress transcription, most of them activate transcription (Fujimoto et al., 2000). In *Arabidopisis* it was shown that a single ERF TF, CRT/DRE-BINDING FACTOR1 (CBFI) which controls tolerance to cold, has a major effect on a complex set of stress responses in plants (Jaglo-Ottosen et al., 1998). Abiotic stresses contribute to the activation of ERF genes along with pathogen infection and disease-related stimuli (Xu et al., 2008).

Basic region/leucine zipper motif (bZIP) TFs regulate many processes in plants including seed maturation and flower development, stress and light signaling and pathogen defense. bZIP TF's make up a large family, and in *Arabidopsis* there are about 75 members (Jakoby et al., 2002). A recent study looked at the Maf family of transcription bZIP factors and alternative recognition mechanisms of DNA (Kurokawa et al., 2009).

Controlling many aspects of plant development is the MYB TF family which represents the largest transcription family in Arabidopsis. (Chen et al., 2005). The first plant MYB protein was identified in maize and is known as the maize *colorless*1 (*c*1) gene. Plant MYB TFs respond to stress signals and hormones and are important in cellular morphogenesis and secondary metabolism; most MYB TFs are positive regulators of transcription (Chen et al., 2005). In higher plants the MYB superfamily of transcription factors are responsible for contributing to defense response and regulatory processes (Yanhui et al., 2006b). The analysis of the complete *Arabidopsis* sequence identified 198 genes of the MYB superfamily (Yanhui et al., 2006a). MYB TFs have also been studied in wheat (Chen et al., 2005) and rice (Yanhui et al., 2006a).

Many zinc finger motifs identified in plant proteins have been found in putative TFs. The classes of zinc-finger motifs present in TFs function as parts of protein-protein interaction and DNA-binding domains. Many of the proteins are unique to plants and have been associated with important biological regulatory processes such as light-regulated morphogenesis, flower development and pathogen response (Takatsuji, 1998).

WRKY transcription factors have been widely studied in different pathosystems including *Arabidopsis* pathosystems where they are crucial regulators of the defense transcriptome and disease resistance (Eulgem and Somssich, 2007a). The WRKY TF genes form large families in plants with nearly 100 members in *Oryza sativa,* 72 members in *Arabidopsis* (Eulgem et al.,

2000; Yang et al., 2009) and 37 in the moss, *Physcornitrella patens* (Guo et al., 2008). The WRKY domain contains about 60 amino acids and is the major characteristic of the WRKY family proteins (Eulgem et al., 2000). The conserved heptapeptide WRKYGQK makes up the WRKY motif and it is followed by a type of zinc finger motif C_2H_2 or C_2HC (Eulgem et al., 2000; Yang et al., 2009). WRKY TFs are involved in more than just defense responses (Zhou et al., 1997), for example they participate in sugar signaling in barley (Sun et al., 2003).

MADS box TF's are an important family of regulatory genes (Parenicova et al., 2003) responsible for controlling floral organ development (West et al., 1997). Members of the MADS-box family are recognized for their involvement in developmental processes (Parenicova et al., 2003). MADS box TFs can be found in plants, animal and fungi and each have a conserved DNA-binding domain (Alvarez-Buylla et al., 2000). The complete analysis of the Arabidopsis genome sequence produced 107 genes encoding MADS box proteins (Parenicova et al., 2003).

Many of the TFs mentioned regulate downstream defense responses and are themselves regulated by phosphorylation (Eulgem et al., 2000). Downstream defense responses are activated because of the production of signaling compounds such as reactive oxygen intermediates (ROIs), ethylene (ETH), jasmonic acid (JA), salicylic acid (SA) and nitric oxide (NO) (Wan et al., 2002). Transcriptional activation of pathogenesis-related genes forms part of the plant defense mechanism; these genes encode lytic enzymes (chitanases, glucanases, and proteases), anti-microbial defensins and enzymes for biosynthesis of anti-microbial secondary metabolites called phytoalexins (Nürnberger et al., 2004)

In the recently sequenced genome of soybean, 5,671 putative soybean transcription factor genes, distributed in 63 families, were identified. This represents 12.2% of the 46,430 predicted soybean protein coding sequences (Schmutz et al., 2010). These TFs are similar in sequence to many of the ones already described for *Arabidopsis* and other plants except that their distributions in the different plants vary.

2.4 Soybean-*P. sojae* pathosystem

Soybean (*Glycine max* (L.) Merrill) is the world's most important leguminous crop due to its high content of high-quality protein for food and animal feed, and its capacity for oil production

used for industrial materials and food. Soybeans have also been identified as valuable for human health (Harada and Xia, 2004).

The soybean [*Glycine max* (L.) Merrill]-*Phytophthora sojae* pathosystem consists of the interaction between the destructive pathogen *P. sojae* and its soybean host. Close communication occurs between the two organisms when a plant and a pathogen come into contact (Wan et al., 2002). Plants respond to the presence of a pathogen with antimicrobial defenses and other stress responses while the pathogen's activities focus on colonization of the host and utilization of its resources (Wan et al., 2002). The original recording of Phytopthora root rot of soybean took place in the US in the early 20th century (Ribeiro et al., 1996). *P. sojae* is an oomycete and members of this phylum cause diseases on many crops and ornamental plants (Moy et al., 2004). *P. sojae* is specific for soybean where it causes destructive root and stem rot of soybean plants (Moy et al., 2004). *P. sojae* causes a soybean yield loss of 1-2 billion dollars annually (Tyler, 2007) and is considered the second most significant yield-suppressing disease in the United States (Jia and Kurle, 2008).

2.5 Resistance in soybean to *P.sojae*

Incorporation of major specific resistance genes or partial resistance through breeding programs into soybean cultivars remain the most effective control measure against *P.sojae* (Jia and Kurle, 2008). Planting resistant cultivars with single resistance genes (*Rps* genes) has been the primary strategy for managing *Phytopthora* root rot and stem rot (Gordon et al., 2007). When *Rps* genes are integrated into soybean cultivars and geographically widely deployed, the *Rps* genes have an effective "life" ranging from 8-15 years (Dorrance et al., 2003b) before being overcome by genetic changes in pathogen populations. In soybean at least 14 disease resistance genes against *P. sojae* have been identified (Burnham et al., 2003b) and mapped to eight different loci (Gordon et al., 2007). Out of the 14 disease resistance genes, complete resistance is identified in 13. Eight resistance (*Rps*) genes have been deployed into cultivars so far (Jia and Kurle, 2008). Single alleles may be adequate in providing disease management; but as new virulence pathotypes of *P. sojae* evolve their ability to defeat deployed *Rps* increases (Schmitthenner, 1985; Schmitthenner et al., 1994; Dorrance et al., 2003a). Quantitative resistance, however, is known to be more durable as it is controlled by multiple genes and exerts less selective pressure on the pathogen.

Tooley and Grau (Tooley and Grau, 1982) propose that this type of resistance works by limiting the lesion growth rate of the pathogen in host tissues, which in turn limits yield losses (Burnham et al., 2003a). The extent to which durable or non host or partial resistance involves genetic components that are distinct from R genes remain unclear (Vega-Sánchez et al., 2005). In their study, Vega-Sanchez and associates demonstrated that increased levels of transcript levels for the matrix metalloproteinase, PR-1a, basic peroxidase and glucanase may be associated with partial resistance in infected roots.

2.6 Soybean genomics

The genome of soybean cultivar Williams 82 was recently sequenced. The genome is about 1.1 gigabase. The genome is predicted to have 46,430 protein coding genes and 75% of these genes are present in multiple copies (Schmutz et al., 2010). Prior to the genome sequence, genomic resources available for soybean included 342,359 expressed sequence tags (EST's), a high density genetic map and an extensive physical map.

An accurate and saturated genetic linkage map of soybean aids identification of new Quantitative Trait Loci (QTLs), physical map construction, map-based cloning and whole-genome sequencing (Xia et al., 2008). Over a thousand quantitative trait loci have been mapped in soybean representing ~90 agronomical important traits. Over time, molecular maps have been gradually integrated. The first soybean genetic map was made up of only 57 classical markers. Additional molecular markers consisting of restriction-fragment length polymorphism (RFLP) markers derived from expression sequence tags (EST) were added (Harada and Xia, 2004). Then random amplified polymorphic DNA (RAPD) markers, simple length sequence repeat markers (SSR) and amplified fragment length (AFLP) markers were integrated into the RFLP linkage maps (Harada and Xia, 2004). Recent maps utilize an integrated approach, merging maps from dissimilar mapping populations using software such as JoinMap (Xia et al., 2008).

It is important to integrate trait loci into molecular linkage maps for the cloning of genes responsible for agronomically important traits or map-based cloning, as well as for breeding programs associated with marker assisted selection (MAS) (Harada and Xia, 2004). Trait loci of interest include: flower color (*W1*), color of cotyledon (*D1, D2*), seed coat color(*G, R*) bloom on

seed coat (*B1*), seed coat peroxidase activity (*Ep*), light hilum (*I*), hilum abscission layer (*N*), pod color (*L1*), pubescence color (*T*), density of pubescence (*Pd1, Ps*), morphology of hair tip (*Pb*), glabrousness (*P1*), morphology of leaflet (*Ln*), 5-foliolate leaf (*Lf1*), foliar pigmentation (*Y9*), root fluorescence (*Fr1, Fr2*), indeterminate stem (*Dt1*), flowering and maturity (*E2*) and resistance to bacterial blight (*Rpg1, Rpg4*) all integrated into Cregan's map (1999a) (Harada and Xia, 2004). Also reported are DNA markers linked to resistance genes to soybean mosaic virus, peanut mottle virus, soybean cyst nematode, *Phytophthora* rot, bacterial blight, frogeye leaf spot and downy mildew (Harada and Xia, 2004).

2.7 *P. sojae* genomics

In collaboration with the DOE Joint Genome institute (JGI), the draft genome of *P. sojae* was completed in 2006 (Tyler et al., 2006b). This represents a nine-fold coverage of the 95 Mb *P. sojae* genome (Tyler et al., 2006a) . Over 19,000 predicted genes were identified in the *P. sojae* genome. These included, amongst others, different classes of potential infection-related genes: effectors, hydrolases, protein inhibitors and protein toxins. Other genomic resources available for *P. sojae* include 32,000 ESTs, which include soybean infection ESTs and also physical maps (Wu et al., 2004; Torto-Alalibo et al., 2007). Two Bacterial Artificial Chromosome (BAC) libraries were constructed by restriction enzyme fingerprinting and were used to generate a physical map of *P. sojae* (Zhang et al., 2007). In *P. sojae* at least 12 avirulence genes have been genetically identified (Tyler, 2007).

Another resource available to the scientific community is the Soybean Affymetrix GeneChip. In addition to over 37,000 probe sets derived from the host soybean, this chip also contains over 15,000 probe sets from *P. sojae* and over 7,500 from the cyst nematode *Heterodera glycines*. The presence of probes from these important pathogens on the same chip enables researchers to understand how the soybean plant interacts with two of its key pathogens.

2.8 Microarray Analysis of Host-Microbe Interactions

Microarray measurements in principle allow the estimation of target RNA abundance at the molecular and cellular level and the detection of transcriptional responses to biological interactions (Shiu and Borevitz, 2006). The methodology can be used to analyze gene expression over time, monitoring expression levels of thousands of genes simultaneously (Conesa et al., 2006)**,** enabling overall transcript patterns to be probed under experimental conditions such as infection. Thus, microarray-based studies of transcript levels during plant-pathogen interactions have proven very valuable (Moy et al., 2004) and have been applied to study plant-pathogen interactions and downstream defense signaling (Wan et al., 2002).

The technology for microarrays have grown tremendously since the introduction of the technology used for gene expression research (Schena et al., 1995). The first microarray technology base based on DNA spotted arrays (Schena et al., 1995). Oligonucleotide based arrays and cDNA microarrays are the two widely used methods when discussing the technology of DNA microarrays (Wan et al., 2002). The Affymetrix GeneChip® Soybean Genome Array, was used in this research to study differentially expressed genes contributing to quantitative resistance. The GeneChip was designed by Affymetrix and in collaboration with the Soybean Research Community as part of the GeneChip® programThe gene chip contains 37,500 *G.max* transcripts as well as over 23,3000 transcripts from two additional and important pathogens of soybean, *Heterodera glycines and P. sojae* (Affymetrix, Santa Clara, CA).

In plant microbe interactions, microarrays have been used to study the global expression profiling of diverse pathosystems (Maleck et al., 2000): defense responses of *Brassica napus* (rapeseed) against *Sclerotinia sclerotiorum* (Zhao et al., 2007), interaction of *Arabidopsis thaliana* with *Hyaloperonospora parasitica* (Eulgem et al., 2007), and the potato-*Phytophthora infestans pathosystem* (Tian et al., 2006) soybean-*Herterodera glycines* (Ithal et al., 2007) and soybean-*Phakopsora pachyrhizi* (Panthee et al., 2007).

Genome-wide programming of transcription has been described for plant responses related to basal resistance following bacterial infection of *Arabidopsis* (Joosten and de Wit, 1999; Truman et al., 2006). Microarrays were also used to reveal the gene regulation in potato during infection (Pinzon et al., 2009).

Global expression profiling experiments have been used to help understand major differences between the basal defense, SAR, ETI, and PTI (Eulgem and Somssich, 2007b). It is understood these response mechanisms are not qualitative but quantitative and/or temporal (Eulgem and Somssich, 2007b).

Soybean researchers around the world are using soybean macro-and microarrays to generate expression data for thousands of genes under different experimental conditions. Using cDNA microarrays, Brechenmacher and associates monitored gene expression in soybean in response to the nodulating bacterium *Bradyrhizobium japonicum* and showed that *B. japonicum,* which establishes a mutualistic association with the host soybean actually reduces plant defense during nodulation (Brechenimacher et al., 2008). Alkharouf and associates, using a cDNA microarray platform demonstrated differential gene expression in a susceptible soybean cultivar challenged wth *Heterodera glycines* over a time course (Klink et al., 2007). A recent study also focusing on the cyst nematode *Heterodera glycine*-soybean pathosystems, but using the Affymetrix soybean GeneChip looked at the global transcript changes occurring in the syncitium in a resistant soybean cultivar. In this study, the authors identified genes differentially expressed during the transition from resistant to the parasitism phase (Klink et al., 2009). In another study using the Affymetrix soybean GeneChip platform, Zhou et al 2009 demonstrated that, the entire soybean genome undergoes transcriptional changes in response to infection by *P. sojae*. Observing this phenomenon was made possible by the use of a large number of biological replicates (Zhou et al., 2009)

The soybean genome array also known as the Genechip® Soybean Genome Array was selected to complete the research and study differentially expressed genes.

2.9 Quantitative trait loci associated with disease and defense related genes

Quantitative trait locus (QTL) analysis makes it possible to study multiple genes that contribute to a trait of interest. A QTL is a chromosomal region that is inferred to contain a gene (or cluster of genes) that contributes to the variation observed in a quantitative trait such as disease resistance. QTL mapping studies have been carried out for more than 90 distinct traits of soybean including disease resistance, seed quality and nutritional traits, plant developmental and

reproductive characters (Schmutz et al., 2010)**.** QTL analysis has been applied to a wide variety of plant pathogen interactions including soybean resistance to *P. sojae* (Burnham et al., 2003a) and to nematodes (Guo et al., 2006). QTLs for resistance have been analyzed for soybean diseases and pests such as: Sclerotinia stem rot, sudden death syndrome, peanut root-knot nematode, southern root-knot nematode and corn earworm (Harada and Xia, 2004).

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Online Reference

The joint Affimetrix GeneChip http://www.affymetrix.com

Chapter 3

Methods

3.1 Plant Materials and Pathogen Isolate

Four soybean lines with varying degrees of quantitative resistance to *P. sojae* were used to identify regulatory factors. The soybean lines were: cultivar 'Conrad, that has a high level of quantitative resistance to *P. sojae* and is considered the industry standard; 'V71-370', a breeding line with a high level of quantitative resistance; cultivar 'Sloan' that is highly susceptible; and 'VPRIL-9' (VP9) that is a susceptible F12 recombinant inbred line derived from a cross of V71-370 to the *Glycine soja* line PI407162 (Burnham et al., 2003). The *P. sojae* isolate PT2004C2.21 (virulent to *Rps*1a, *Rps*1b, *Rps*1k, *Rps*2, *Rps*3a, *Rps*3c, *Rps*4, *Rps*5, *Rps*6 and *Rps*7) was selected because it was unaffected by any of the *Rps* genes present in the soybean cultivars used in the project (Vega-Sánchez et al., 2005; Mideros et al., 2007)

3.2 Inoculation of soybean plants with *P. sojae*

The following procedures were carried out by the project collaborators at the Ohio State University. The slant board technique was used to carry the inoculation of soybean seedlings with *P. sojae* (Burnham et al., 2003). Seedlings, grown seven days in vermiculite, were thoroughly rinsed in tap water and placed in an inoculation tray (20-30 plants per tray). The plants were slightly scraped at 2 cm below the beginning of the root zone and then inoculated with an agar slurry that was sterile or infested with pathogen mycelium. Hypocotyl sections 7.5 or 15 mm long were collected at 1, 2, 3 and 5 days post inoculation (dpi). For infected plants 3 and 5 days post inoculation, hypocotyl sections were collected from 7.5 mm below the lesion margin; this will be referred to as the "Lower" infection court . Samples were also taken 7.5 mm above the lesion margin; these will be designated as the "Upper" infection court. For mock inoculated plants, 15 mm hypocotyl sections were taken spanning a position corresponding to the average lesion length in the inoculated plants. At early time points, 1 dpi and 2 dpi, the lesion was not usually visible. Because of this, 15 mm hypocotyl sections were collected that spanned the site of inoculation (mock or with pathogen. Two sets of 30 plants grown in different places in the same growth chamber were sampled for each treatment and equal amounts of RNA from the

two sets of 30 were pooled. The whole experiment was replicated four times. All plants were grown under the same conditions in a growth chamber (Chargrin Falls, Model M-48 with TC2 microcontroller unit). Day and night temperature settings remained at 27°C and 21°C, and relative humidity averaging 75% to 90%.

3.3 RNA extraction and Microarray assays

The following procedures were carried out by the project team (including Lachelle Waller) and the Core Laboratory (CLF) at the Virginia Bioinformatics Institute. For total RNA isolation from pathogen, or mock, inoculated soybean tissue sections, the QIAGEN RNeasy® Plant Mini Kit was used as directed by the manufacturer with slight modifications (Zhou et al., 2009). The Agilent 2100 Bioanalyzer was used to check the quality of total RNA. Equal amounts of RNA samples were pooled from the two inoculation replications (see previous section) and sent to the CLF (http://www.vbi.vt.edu/core_laboratory_facility) where microarray procedures were performed following the standard eukaryotic expression assay protocols described in the Affymetrix Gene Chip® Expression Analysis Technical Manual. To generate the biotin-labeled cRNA, 1 μg of total RNA was used. The hybridization was performed at 45°C for 16 h in an Affymetrix hybridization oven (model 640), and then the GeneChips were washed and stained with streptavidin-phycoerythrin prior to scanning.

3.4 Low Level Data Processing

The lower level data processing included quality control, filtering and GCRMA. These procedures were carried out by the project's statistical team at the Virginia Bioinformatics Institute.

3.4.1 Quality Control

The Quality control was done using various tools from the Bioconductor, Affymetrix Microarray Suite version 5 (MAS5) and AffyPLM quality control toolsets. To begin the low-level analysis, the raw GeneChip data were analyzed by MAS5 gene filtering protocols to identify expressed ("present") and non-expressed ("absent") genes using a ScaleT (tau) setting of 0.006. The tau value is the cutoff used to describe the variability of the probe pairs in the probe set (Affymetrix white paper 2002). (the Bioconductor package *affy*, http://bioconductor.org/packages/2.0/bioc/html/affy.html provides implementation). A gene

was defined as detectable if it was called “Present” in all four replicates in at least one of the 40 assayed conditions. Out of the total 37,593 soybean genes represented by probe sets on the Affymetrix soybean GeneChip, 30,166 genes were identified with detectable expression under our experimental conditions.

3.4.2 Data Pre-processing

To normalize the gene expression values the primary method used for data pre-processing was GeneChip Robust Multi Array (GC-RMA). The background subtraction was completed via the “gcrma” bioconductor package. All data was normalized using an implementation of the GC-RMA quantile normalization procedure written in C code by Bao et al (unpublished).

The summarized expression values of detectable genes were generated using the median polish procedure, implemented in the R environment for statistical computing.

3.5 High Level Data processing

The following procedures were carried out by Lachelle Waller

3.5.1 Linear Mixed Model Analysis (LMMA) of Gene Expression Levels

Linear Mixed Model Analysis is a procedure for estimating the contributions of both fixed and random effects to the variation observed in a set of repeated measurements. In this case the measurements (or response variable) are the gene expression values as summarized by GCRMA, on logarithmic (base 2) scale. The fixed factors include cultivar treatment (inoculation or mock) and time and place of sampling. The random effect is variation among the experimental replicates.

To complete the LMMA analysis, the PROC MIXED procedure was used from the Statistical Analysis System (SAS 9.1 for Windows, SAS institute., Cary, NC, USA) (Nettleton, 2006). All fixed factors were considered as a family. The Satterthwaite approximation for the denominator degrees of freedom was used (Zhou et al., 2009).

3.5.2 False Discovery Rate (FDR) control

There is a potentially high rate of false positives when completing microarray studies because tens of thousands of hypotheses are being tested simultaneously (Pawitan et al., 2005). Three

methods were evaluated for controlling the false discovery rate (FDR), the relatively conservative linear step up method of Benjamini and Hochberg (Benjamini and Hochberg, 1995), the moderately conservative two-stage linear step-up procedure (TST-FDR) (Benjamini et al., 2006), and the relatively permissive Q-value method described by Storey and Tibshirani (Storey and Tibshirani, 2003) that uses the p-values to compute the q-values for the positive FDR measure. Most of the results reported in this thesis were obtained with the TST method.

3.5.3 Contrast analysis

The LMMA contrast analysis using SAS Proc Mixed was used to detect various categories of expression differences (Zhou et al., 2009). The first contrast was the constitutive contrast (CC) which observes the difference between constitutive expression levels in two different cultivars under mock-inoculation conditions. The second contrast was the infection contrast (IC) which compares the gene expression levels between two cultivars following infection. The third contrast was the infection response (IR) which compares gene expression in one particular cultivar during pathogen infection to that following mock inoculation. The fourth contrast was the response contrast (RC) which compares the infection response between two cultivars.

All contrasts in a data series were considered as a family. FDR was used as the control method for the family at a level of 0.05, using the TST-method (Reiner et al., 2003). The upper infection court data series ("TimeU") included all samples except the "Lower" treatments of 3 and 5 days post inoculation. Similarly, the lower infection court data series ("TimeL") included all samples from all time points, except for the "Upper" treatments of 3 and 5 days post inoculation.

Results from different time points were investigated via Boolean comparison. To better understand the biological relevance of those genes that showed significant contrasts, the "Gene report" database (see below), Perl scripts and open source software such as BioConductor packages in the R (bioconductor.org) environment were used.

3.5.4 Identification and mapping of potential regulatory genes.

To identify regulatory factors among the differentially expressed genes, annotations provided by the Goldberg group at the University of California, Los Angeles (http://estdb.biology.ucla.edu/seed) were used, together with the annotations provided by

Affymetrix. Known transcription factor databases and information from the scientific literature was also used to identify putative transcription factors and other regulatory genes.

To identify regulatory factors potentially contributing to quantitative resistance, the map positions of the genes encoding the factors were compared to those of known phenotypic QTL that control *P. sojae* resistance in soybean(Tucker et al., 2010). The map positions of the genes were determined by matching the sequence of the gene to the soybean genome sequence, and then matching that portion of the genome sequence to a high density SFP map of soybean (Krampis, 2010)

To carry out the mapping, text files were created using the gene identification (gene ID) for each gene of interest. The text files were uploaded into the NetAffx Analysis Center (affymetrix.com). A BLAST search of selected gene sequences were done against the soybean scaffolds available on the Phytozome database (www.phytozome.org). The scaffolds were matched to a high density map created using single feature polymorphisms (SFP) (Krampis, 2010) and phenotypic QTL data that control resistance in soybean (Tripathy, Bao, Tucker et al; unpublished data).

3.6 Database

The following procedures were carried out by Lachelle Waller.

The database is also known as the "Gene Report" application and is made up of two different components. The user is anyone who is interested in analyzing a scenario or series of experiments. Specifically anyone interested in time course data and if genes are differentially expressed, up-regulated and or down-regulated. The database is useful for others because it allows individuals the ability to deposit time course data, pertaining to a given scenario or series of experiments. What information needs processing is important. For example Affymetrix data can be loaded into the database from the front end of the database after identifying the specific field of interest. An individual would have to define the scenario across their data or any data matching the fields of data in the database. For this thesis, "Gene Report" contained four cultivars of varying partial resistance to *P. sojae* together with soybean gene expression data over four time points. A realistic example of the database is similar to an individual having the

ability to upload his or her data to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) or NCBI (http://www.ncbi.nlm.nih.gov/).

To test if the system worked it was important to manually double check the original text files in Excel.

All data uploaded to the database was normalized, see section 3.2, and only those specific fields of interest needed to complete the analysis were filled.

3.6.1 Development

The "Gene Report" application has an interface design and was developed using XHTML and CSS (cascading style sheets). Originally the data was exported to comma separated values (csv) text files. At that point the data was moved to SQlite which can be used to store the gene information locally. In order to improve response time for a web interface the database was moved to a server using MySQL. The MySQL database including tables' views and functions is a complete copy of the aforementioned SQlite database. The back end functionality has one class that serves to just retrieve the gene information and combine it with the test data. Fig. 1.1 provides an example of each field required for each table. There were two tables used to analyze the data. Two classes were created. Class one, called the gene, is a table in which all information about each gene was stored from the UCLA and Affymetrix annotations. The file contained all annotation information and functional category information available from Affymetrix for each probe set. The UCLA information was obtained by BLAST searches against known databases such as The Arabidopsis Information Resource (TAIR) database, the TIGR Plant Transcript Assemblies (TA) database, and the NCBI non-redundant database. For gene sequences which did not have sequence similarity to any proteins in the public databases to the public, functional groups were manually assigned based on available literature. All gene sequences with unknown functions were classified as Unknown or Unclassified Function (Brandon Le) (http://estdb.biology.ucla.edu/seed). Additional information regarding the annotations can be found at (http://seedgenenetwork.net/media/Soybean_Array_Annotation_Su.pdf).

Two different databases were created and used to store and analyze the results of the contrast analyses (see Methods section 3.5.3) presented in chapter four and chapter five. To analyze all genes of interest in chapter four, RUBY was used as the programming language. A flat file

database was used as the database. Ruby was used because of its strength in parsing text. All csv text files were flattened into one flat file within the database. This included the UCLA file containing annotations and functional group information (http://estdb.biology.ucla.edu/seed).

To analyze all genes of interest in chapter five, a MySQL database was used with the schema shown in Figure 3.1.

The database should be useful for others because it allows individuals the ability to deposit time course data, with a given scenario or series of experiments and have the ability to query whether genes of interest are up regulated or down regulated. It is also useful for others because it provides an example of how to set up websites.

Figure 3.1. Database table designs, including which fields go into each table

```
CREATE TABLE [data]
(
 [contrastID] VARCHAR(32) NOT NULL,
 [geneID] VARCHAR(32) NOT NULL, -- From "Transcript ID" in
Gm37593_bx_annot_10_30_05.txt
 [p] FLOAT NULL,
 [adjpBH] FLOAT NULL,
 [significantBH] BOOLEAN DEFAULT 0 NOT NULL,
 [adjpTST] FLOAT NULL,
 [significantTST] BOOLEAN DEFAULT 0 NOT NULL,
 [qvalue1] FLOAT NULL,
 [significantQ1] BOOLEAN DEFAULT 0 NOT NULL,
 [qvalueS] FLOAT NULL,
 [significantQS] BOOLEAN DEFAULT 0 NOT NULL,
 [qvalueB] FLOAT NULL,
 [significantQB] BOOLEAN DEFAULT 0 NOT NULL,
 [t] FLOAT NULL,
 [Estimate] FLOAT NULL,
 [FoldChange] FLOAT NULL,
 [orientBH] INTEGER NULL,
 [orientTST] INTEGER NULL,
 [orientQ1] INTEGER NULL,
 [orientQS] INTEGER NULL,
 [orientQB] INTEGER NULL,
 [day] INTEGER NOT NULL,
 [contrast] VARCHAR(2) NOT NULL,
 [cultivar1] VARCHAR(3) NOT NULL,
```

```
 [cultivar2] VARCHAR(3) NULL,
 UNIQUE([contrastID],[day],[contrast],[cultivar1],[cultivar2],[geneID]),
 FOREIGN KEY ([geneID]) REFERENCES [gene] ([gid])
);
;
```

Figure 3.1 (A) Table (Data) design, including all information required in each field. This table contained the information required as user input.

```
CREATE TABLE [gene]
(
[gid] VARCHAR(32) NOT NULL, -- From "Probe ID" in
Soybean_GeneChip_Annotation_Updated_Oct2007.csv
[genebankid] VARCHAR(32) NULL, --  From "GenBankID" in
Soybean_GeneChip_Annotation_Updated_Oct2007.csv
[TIGR] VARCHAR(512) NULL, --  From "TIGR (PlantTA) Description" in
Soybean_GeneChip_Annotation_Updated_Oct2007.csv
[ath1] VARCHAR(32) NULL, --  From "ATH1 Probe ID" in
Soybean_GeneChip_Annotation_Updated_Oct2007.csv
[agi] VARCHAR(32) NULL, --  From "New AGI ID" in
Soybean_GeneChip_Annotation_Updated_Oct2007.csv
[arabidopsis] VARCHAR(512) NULL, --  From "Arabidopsis" in
Soybean_GeneChip_Annotation_Updated_Oct2007.csv
[functionalcat] VARCHAR(512) NULL, -- From "Functional Category (2007)" in
Soybean_GeneChip_Annotation_Updated_Oct2007.csv
[subcategory] VARCHAR(512) NULL, -- From "Sub-Category (2007)" in
Soybean_GeneChip_Annotation_Updated_Oct2007.csv
[family] VARCHAR(128) NULL, -- From "Family (2007)" in
Soybean_GeneChip_Annotation_Updated_Oct2007.csv
UNIQUE ([gid])
);
;
```

Figure 3.1 (B) Table (Data) design, including all information required in each field. This table contained the information required as user input to attach the annotations and functional groups. All data received from UCLA soybean data set. 2007 Soybean GeneChip Annotation last updated October 2007

Although we began analyzing selected genes in chapter four with MySQL we realized later that using Ruby text processing was acceptable to complete the data analysis in chapter four and actually ran much faster.

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http://www.ncbi.nlm.nih.gov/

http://estdb.biology.ucla.edu/seed

(http://seedgenenetwork.net/media/Soybean_Array_Annotation_Su.pdf)

Chapter 4

Transcriptional responses of soybean genes during infection of cultivars with different levels of quantitative resistance by *Phytophthora sojae*

4.1 Introduction

Genetic strategies to control *P. sojae* have focused both on the use of varieties with single *Rps* resistance genes and on varieties with partial or quantitative resistance that is more durable in the presence of evolving pathogens. Although varieties with partial resistance have been used in breeding programs and in production, the molecular mechanisms underlying this type of resistance are not fully understood. Microarray technologies have been applied as a step to unravel global expression genes in plants under varying conditions, including pathogen challenge (Huitema et al., 2003). Genetic analysis of partial resistance in the greenhouse and in the field has been used to identify and map QTLs. Furthermore, ESTs have been generated from infected tissue and have provided information on host and pathogen transcripts associated with infection in the *P. sojae*-soybean pathosystem and also in the *P. infestans* potato and tomato pathosystems. However no study has yet examined global gene expression over a time course in the context of partial resistance to *P. sojae*. This chapter describes the transcriptional responses of soybean during the course of infection with *P. sojae* using four cultivars with different levels of partial resistance.

4.2 Results

We examined soybean cultivars Conrad, V71-370, VPRIL-9 (VP9) and Sloan inoculated with the *P. sojae* isolate PT2004C2.S1. Conrad and V71-370 were previously characterized as having partial resistance against *P. sojae*, while VP9 and Sloan were characterized as susceptible (Burnham et al., 2003). RNA was isolated from the hypocotyls of seedlings challenged with the *P. sojae* isolate. Inoculated hypocotyl sections were collected after 1, 2, 3 and 5 days post

inoculation (dpi). We chose this particular strain of *P. sojae* because it is compatible with all *Rps* genes present in the selected soybean cultivars used in the experiment (Vega-Sánchez et al., 2005). For pathogen-inoculated hypocotyls at 3 dpi and 5 dpi, 7.5 mm long samples were taken immediately above and immediately below the lesion margin. Thus the lower sample was from symptomatic tissue and the upper samples were from non-symptomatic tissue. At 1 dpi and 2 dpi, the 15 mm region spanning the inoculation site was sampled; in most cases this was still asymptomatic at 2 dpi. When 1 or 2 dpi data were compared with 3 or 5 dpi data, the 3 or 5 dpi data from the upper, asymptomatic tissue were used as being more comparable with the asymptomatic 1 and 2 dpi tissue samples. The experiment was biologically repeated four times. All data from the 128 individual GeneChips (1 dpi, 2 dpi, 3 dpi upper, 5 dpi upper; inoculated and mock; 4 cultivars; 4 replicates) were normalized using GC-RMA, and MAS5 was used to identify genes with a detectable transcript level. This resulted in the identification of 30,166 genes with detectable transcripts.

Genes responding to infection were identified in each cultivar and classified according to the time points (1dpi, 2 dpi, 3 dpi and 5 dpi) for which they were specific. To identify transcripts that had statistically significant variation in abundance in response to infection, contrast analysis using Linear Mixed Model Analysis of Variance (LMMA) was used. The false discovery rate was controlled at the 0.05 level using the TST-FDR method. BLASTX annotations of genes were taken from The Soybean Genome Project, DoE Joint Genome Institute (http://www.soybase.org/SoyBase/AffyChip/index.php). Functional categories were based on assignments provided by the Goldberg group at the University of California, Los Angeles (http://estdb.biology.ucla.edu/seed) (note some genes were annotated differently by the JGI and UCLA groups).

The numbers of genes up-regulated exclusively on day 1 in the four cultivars in response to *P. sojae* challenge are shown in Figure 4.1.

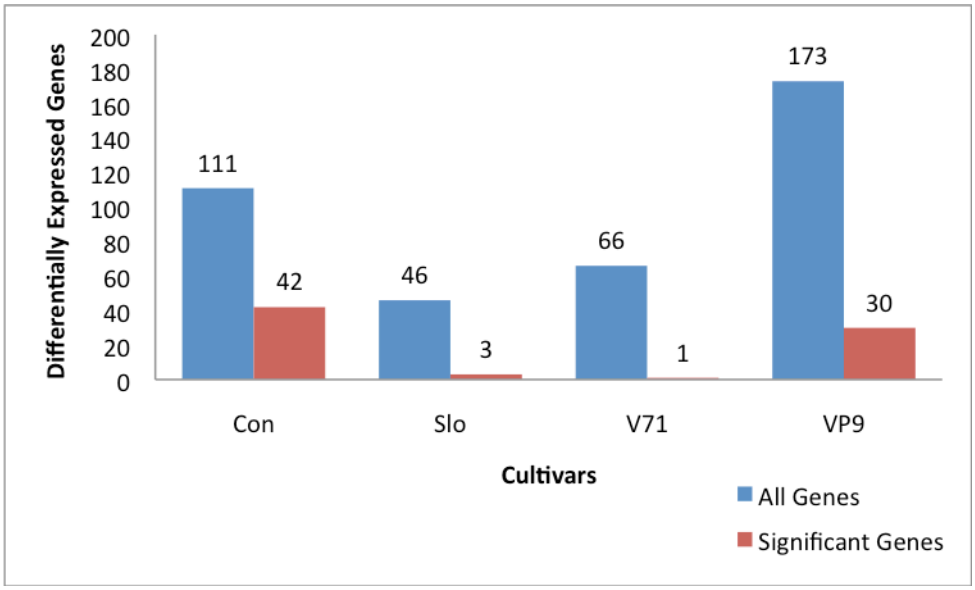


Figure 4.1. Differentially expressed genes up-regulated exclusively at 1 day post inoculation.

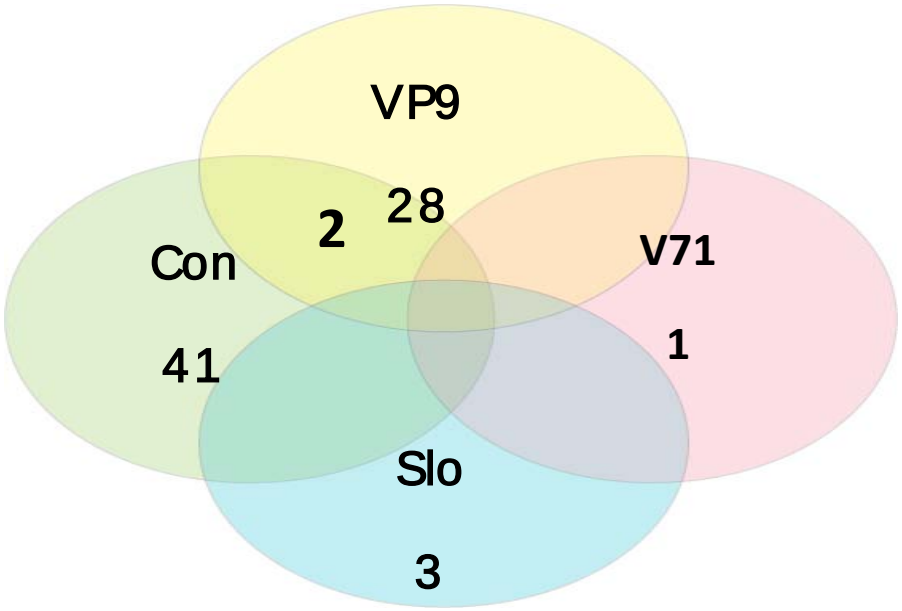


Figure 4.2 Venn diagram of the four cultivars and the number of differentially expressed genes identified in each cultivar. Resistant cultivars (Con and V71) and susceptible cultivars (VP9 and Slo)

While only 1.5% of genes up-regulated exclusively at 1dpi in the most resistant cultivar (V71-370) were significantly up-regulated, 37.8% of up-regulated genes were significantly up-regulated in another relatively resistant cultivar Conrad. Similarly, 6.5% and 17.3% of the up-regulated genes were significantly up-regulated in the susceptible cultivars Sloan and VPRIL9

respectively. The 42 genes significantly up-regulated in Conrad included five involved in signal transduction, four in metabolism, three in transcription, one associated with disease and defense and 21 with no known functions (Table 4.1). In Conrad a gene, GmaAffx.51578.1.S1_at, similar to *Arabidopsis* hypothetical protein At5g25170 and identified in 'Disease and Defense' had an ortholog that was identified in a study to understand drought tolerance in cassava as a gene unique to the dehydration and stress (Lokko et al., 2007). In V71-370 the only up-regulated transcript, GmaAffx.79484.1.A1_at, was annotated as a Cyclin-dependent kinase inhibitor 1;2.

Table 4.1. Functional classification of genes significantly up-regulated exclusively at 1 dpi in Conrad

Gene ID	Annotation	Functional Categories	Infection response (fold change) *
Gma.9086.1.S1_at	Cellulose synthase	Cell Growth & Division	1.42
Gma.6130.1.S1_at	T25N20.22 Arabidopsis_thaliana	Cell Structure	1.21
GmaAffx.49343.1.A1_at	Putative beta-d-xylosidase	Cell Structure	1.58
GmaAffx.51578.1.S1_at	Hypothetical protein At5g25170	Disease & Defense	1.38
Gma.3568.2.S1_at	At5g10260	Intracellular Traffic	1.34
GmaAffx.33147.1.S1_at	Cytochrome P-450	Metabolism	1.79
GmaAffx.36153.1.S1_at	Hypothetical protein F15N18 130	Metabolism	1.35
Gma.16606.2.S1_a_at	SOS2 like protein kinase	No Homology to Known Proteins	1.34
Gma.6451.2.S1_s_at	Function unknown	No Homology to Known Proteins	1.24
Gma.9774.1.S1_at	Function unknown	No Homology to Known Proteins	1.23
GmaAffx.2735.2.S1_at	Hypothetical protein	No Homology to Known Proteins	2.21 R
GmaAffx.28001.1.S1_at	Hypothetical protein	No Homology to Known Proteins	1.32
GmaAffx.43001.1.S1_s_at	Function unknown	No Homology to Known Proteins	1.58
GmaAffx.75617.1.A1_at	Function unknown	No Homology to Known Proteins	1.56
GmaAffx.78925.1.A1_at	Function unknown	No Homology to Known Proteins	1.18
Gma.16336.1.S1_at	Putative methionine sulfoxide reductase B	Protein Destination & Storage	1.25
Gma.8301.1.S1_at	Function unknown	Protein Destination & Storage	1.3
GmaAffx.2999.1.A1_at	O-methyltransferase	Secondary Metabolism	1.61
Gma.1007.2.S1_at	Putative calmodulin related protein	Signal Transduction	1.6
Gma.16891.1.S1_at	Hypothetical protein At3g05010	Signal Transduction	1.29
Gma.17368.1.S1_at	Serine/threonine kinase	Signal Transduction	1.43
Gma.3633.1.S1_at	Function uknown	Signal Transduction	1.46
GmaAffx.31978.1.S1_at	Integrase, catalytic region; Zinc finger, CCHC-type; Peptidase aspartic, catalytic	Signal Transduction	1.25
Gma.15643.1.A1_x_at	MYB transcription factor MYB129	Transcription	1.65

Gma.4207.2.S1_at	MYB transcription factor MYB48	Transcription	1.51
GmaAffx.1166.1.S1_at	AOBP	Transcription	1.7
GmaAffx.84261.1.S1_at	Transcription factor like protein	Transcription	1.35
Gma.18052.1.S1_at	Protein At2g28315	Transporter	1.31
GmaAffx.68354.1.S1_at	Probable sulfate transporter 3.3	Transporter	2.03
Gma.12227.1.S1_at	Function unknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.55 R
Gma.1498.1.A1_at	TRNA-binding arm; Pleckstrin homology type	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.48
Gma.445.1.A1_at	Function unknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.25
Gma.8234.1.S1_at	Hypothetical protein At1g65000/F13O11 34	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.3
GmaAffx.48977.1.S1_at	Function unknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.6
GmaAffx.90674.1.S1_x_at	Hypothetical protein P0437H03.128	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.38
Gma.10800.1.S1_s_at	Light harvesting protein	Unclassified - Hypothetical Protein Supported by cDNA	1.16
Gma.1443.1.S1_at	Expressed protein	Unclassified - Hypothetical Protein Supported by cDNA	1.36
Gma.16711.1.S1_at	Function unknown	Unclassified - Hypothetical Protein Supported by cDNA	1.36
Gma.6725.1.S1_at	Function unknown	Unclassified - Hypothetical Protein Supported by cDNA	1.22
GmaAffx.20108.1.S1_at	Hypothetical protein At5g22090/T6G21 200	Unclassified - Hypothetical Protein Supported by cDNA	1.65
GmaAffx.2745.2.A1_at	Function unknown	Unclassified - Hypothetical Protein Supported by cDNA	1.41
Gma.7807.1.A1_at	Hypothetical protein At3g02720	Unclassified - Protein with Unknown Function	1.3

* **Genes also identified as up-regulated in susceptible line VPRIL-9 are indicated by "R"**For the relatively susceptible line, VPRIL-9, genes significantly up-regulated included three that are associated with disease and defense, three with metabolism, one with signal transduction, three with transcription, and 19 with unknown functions (Table 4.2). Interestingly the 3 genes significantly up-regulated in the most susceptible cultivar Sloan (Table 4.3) do not overlap with any up-regulated in VPRIL-9.

Table 4.2. Functional classification of genes significantly up-regulated exclusively at 1 dpi in VPRIL-9

Gene ID	Annotation	Functional Categories	Infection response (fold change) *
Gma.131.1.S1_at	Peroxidase	Disease & Defense	1.54
Gma.8522.1.S1_at	Major latex protein homolog	Disease & Defense	1.77
GmaAffx.73174.1.S1_at	Protein At4g14723	Disease & Defense	1.64
Gma.4408.2.S1_at	NADP-dependent malic enzyme	Metabolism	1.36
Gma.4523.1.S1_at	Alpha glucosidase- like protein	Metabolism	1.21
GmaAffx.9717.1.A1_at	Plastid alpha-amylase	Metabolism	1.25
Gma.15685.1.A1_at	Function unknown	No Homology to Known Proteins	1.56
GmaAffx.2735.2.S1_at	Hypothetical protein	No Homology to Known Proteins	2.06 C
GmaAffx.51464.1.S1_at	Function unknown	No Homology to Known Proteins	1.41
GmaAffx.87414.1.S1_at	Function unknown	No Homology to Known Proteins	1.55
Gma.1100.1.S1_at	F18O14.3 Arabidopsis_thaliana	Protein Destination & Storage	1.36
Gma.7190.2.S1_a_at	Phytochrome B	Signal Transduction	1.33
Gma.16640.2.S1_a_at	Ttg1-like protein	Transcription	1.96
Gma.6619.1.S1_at	Function unknown	Transcription	1.3
GmaAffx.87860.1.S1_at	MYB transcription factor MYB51	Transcription	1.26
Gma.12227.1.S1_at	Function unknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.6
Gma.13241.1.S1_at	Function unknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.63
Gma.16984.1.A1_at	Function unknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.71
Gma.6415.1.S1_at	Yippee-like protein	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.22
GmaAffx.9069.1.S1_at	Function unknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.43
Gma.1451.1.S1_at	Expressed protein	Unclassified - Hypothetical Protein Supported by cDNA	1.36
Gma.16711.2.S1_at	Function unknown	Unclassified - Hypothetical Protein Supported by cDNA	1.16
Gma.4078.1.S1_at	Function unknown	Unclassified - Hypothetical Protein Supported by cDNA	1.3
Gma.7135.1.S1_a_at	Function unknown	Unclassified - Hypothetical Protein Supported by cDNA	1.35
Gma.9207.1.S1_at	Hypothetical protein P0435H01.7	Unclassified - Hypothetical Protein Supported by cDNA	1.27
GmaAffx.3703.1.S1_at	Engulfment and cell motility	Unclassified - Hypothetical Protein Supported by cDNA	1.24
GmaAffx.37697.1.S1_at	Hypothetical protein	Unclassified - Hypothetical Protein Supported by cDNA	1.48
GmaAffx.88692.1.S1_s_at	Function unknown	Unclassified - Hypothetical Protein Supported by cDNA	1.67
Gma.17207.1.S1_at	Function unknown	Unclassified - Protein with Unknown Function	1.46
Gma.423.1.S1_at	Remorin C-terminal region	Unclassified - Protein with Unknown Function	1.52

*** Genes also identified as up-regulated in resistant cultivar Conrad are indicated by "C".**

In the susceptible line VPRIL-9, three transcripts involved in 'Disease and Resistance' were identified: Gma.131.1.S1_at (Peroxidase), Gma.8522.1.S1_at (Major latex protein homolog), and GmaAffx.73174.1.S1_at (Protein At4g14723), together with a MYB transcription factor (GmaAffx.87860.1.S1_at). A study was done to examine the roles of peroxidase with the reactive oxygen species and their reactions generated in plant defense and induction of growth (Kawano, 2003). Peroxidases are involved in numerous physiological processes especially defense mechanisms against the infection of pathogens, wound healing, lignification, auxin catabolism and suberization (Kawano, 2003). Reactive oxygen species (ROS) can cause oxidative damage in excess but make a positive contribution in regulating root growth in response to water stress response (YAMAGUCHI and SHARP, 2010). The major latex protein is associated with pathogen defense responses and with fruit and flowers. It was first studied in opium poppy seed but was also studied in *Arabidopsis* (Lytle et al., 2009). MYB transcription factors are involved in various pathogen or elicitor induced resistance reactions (Eulgem, 2005). They control many aspects of plant development and represent the largest transcription family in Arabidopsis (Chen et al., 2005). Plant MYB TFs respond to stress signals and hormones and are important in cellular morphogenesis and secondary metabolism; most MYB TFs are positive regulators of transcription (Chen et al., 2005).

Table 4.3. Functional classification of genes significantly up-regulated exclusively at 1dpi in Sloan

Gene ID	Annotation	Functional Categories	Infection response (fold change)
GmaAffx.16446.2.S1_at	Similarity to actin	Cell Structure	1.57
GmaAffx.7824.1.A1_at	Hypothetical protein	Transposon	1.36
GmaAffx.9016.1.S1_at	Exostosin-like	Cell Growth & Division	1.79

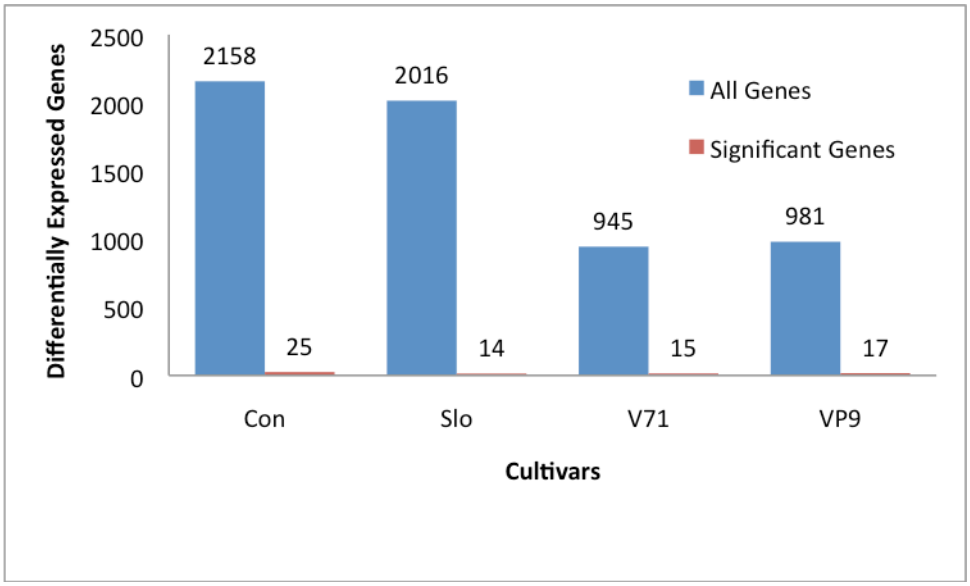


Figure 4.3. Differentially expressed genes up-regulated exclusively at 2 days post inoculation. Resistant lines: V71-370 (V71) and Conrad (Con). Susceptible lines Sloan (Slo) and VPRIL-9 (VP9).

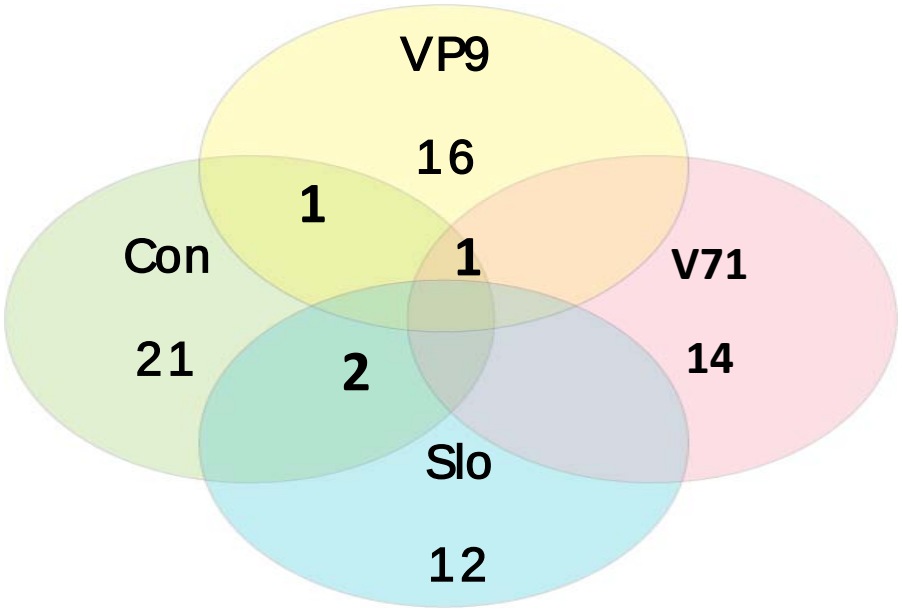


Figure 4.4 Venn diagram of shared differentially expressed cultivars exclusively at 2 day post inoculation. Resistant lines Conrad (Con) and V71-370 (V71) and susceptible lines VPRIL-9 (VP9) and Sloan (Slo)

Relatively more genes were up-regulated exclusively at 2 dpi compared to 1 dpi in all four cultivars (Figure 4.3). Less than two percent of the differentially expressed genes (Figure 2) were found to be significant and up-regulated at day 2 among the four cultivars. The resistant cultivar Conrad contained the highest number of significant differentially expressed genes, while Sloan, the most susceptible cultivar, expressed the least number of significant differentially expressed genes.

Table 4.4. Functional classification of genes significantly up-regulated exclusively at 2 dpi in Conrad.

Gene ID	Annotation	Functional Categories	Infection response (fold change) *
GmaAffx.65693.2.S1_s_at	Phosphoenolpyruvate carboxykinase	Energy	1.89
GmaAffx.90036.1.S1_s_at	Hypothetical protein	Intracellular Traffic	1.27
Gma.15490.1.S1_a_at	Carbonic anhydrase	Metabolism	2.03
GmaAffx.45592.2.S1_at	F9L1.4 protein	Metabolism	1.86
Gma.3828.1.S1_at	Hypothetical protein	No Homology to Known Proteins	5.58
Gma.4818.1.S1_s_at	Function unknown	No Homology to Known Proteins	1.61
GmaAffx.74601.1.A1_at	Function unknown	No Homology to Known Proteins	1.56
Gma.1041.1.S1_at	DnaJ-like protein	Protein Destination & Storage	1.25

Gma.10804.2.A1_s_at	Chaperone protein dnaJ 8, chloroplast precursor	Protein Destination & Storage	2.4
Gma.3473.1.S1_at	Function unknown	Protein Destination & Storage	11.1 R
Gma.7789.2.S1_s_at	Ubiquitin C variant	Protein Destination & Storage	1.54 V
Gma.17655.1.S1_at	Serine/threonine protein kinase pk23	Signal Transduction	1.51
Gma.5758.1.S1_at	Calmodulin-binding protein 60-A	Signal Transduction	1.33
GmaAffx.25698.1.S1_at	F12K21.25	Signal Transduction	2.52
GmaAffx.33050.1.S1_x_at	Calcium-binding EF-hand	Signal Transduction	2.07
Gma.1416.1.S1_at	H-protein promoter binding factor-1	Transcription	2.06
Gma.5293.2.S1_x_at	Putative ethylene response factor 5	Transcription	1.72
Gma.3758.2.S1_at	Function uknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	2.33 S
GmaAffx.82209.1.S1_at	Arabidopsis thaliana genomic DNA, chromosome 5,BAC clone:F24B18	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.52
Gma.2008.1.S1_a_at	Hypothetical protein F27B13.90	Unclassified - Hypothetical Protein Supported by cDNA	1.39
Gma.2034.1.S1_at	F21H2.1 protein	Unclassified - Hypothetical Protein Supported by cDNA	1.21
Gma.2630.1.S1_at	Hypothetical protein	Unclassified - Hypothetical Protein Supported by cDNA	1.48
Gma.6183.1.S1_s_at	Hypothetical protein F15N18 90	Unclassified - Hypothetical Protein Supported by cDNA	1.73
Gma.17498.1.S1_at	Hypothetical protein T22E16.190	Unclassified - Protein with Unknown Function	1.28 S
GmaAffx.91578.1.S1_s_at	Hypothetical protein At3g54190	Unclassified - Protein with Unknown Function	1.39

***** Genes identified as also up-regulated in V71-370 (V) and Sloan (S) or VPRIL-9 (R).

In Conrad, (Table 4.4) the significantly up-regulated transcripts included several relevant to plant defense and classified in “Transcription”, ‘Signal Transduction’ and ‘Protein Destination and Storage”. ‘Signal Transduction’ is important because defense responses in plants are not activated until triggered by host recognition of pathogen encoded molecules (Yang et al., 1997). Calcium signaling is also known to have a relationship mechanism between auxin (Yang and Poovaiah, 2000). A recent study discussed the signaling of auxin mediated by IAA1K a repressor of auxin signaling and a loss of function mutant arf7arf19 (mutant of auxin response factors ARF7 and ARF19) and its involvement with the remodeling of lipids during phosphate starvation (Narise et al., 2010)

Table 4.5. Functional classification of genes significantly up-regulated exclusively at 2 dpi in Sloan.

Gene ID	Annotation	Functional Categories	Infection response (fold change) *
GmaAffx.3897.1.S1_at	Digalactosyldiacylglycerol synthase 1	Metabolism	1.93
GmaAffx.93309.1.S1_s_at	Cytochrome P450 monooxygenase CYP83E8	Metabolism	2.12
Gma.10888.2.A1_at	Ubiquitin ligase	No Homology to Known Proteins	3.48
Gma.16673.1.A1_at	Function unknown	No Homology to Known Proteins	1.51
GmaAffx.1828.1.S1_at	Function unknown	No Homology to Known Proteins	2.18
Gma.10804.1.S1_at	Chaperone protein dnaJ 8, chloroplast precursor	Protein Destination & Storage	3.12
Gma.17451.1.S1_at	Ubiquitin C variant	Protein Destination & Storage	1.73
Gma.17451.1.S1_x_at	Ubiquitin C variant	Protein Destination & Storage	1.83
GmaAffx.19116.1.S1_at	Putative receptor-like protein kinase 2	Signal Transduction	3.34
Gma.12798.1.S1_at	GRAS transcription factor	Transcription	1.72
Gma.3758.2.S1_at	Function unknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	2.37 C
GmaAffx.1301.65.S1_at	Function unknown	Unclassified - Hypothetical Protein Supported by cDNA	4.98
GmaAffx.68372.1.S1_at	Hypothetical protein T17J13.160	Unclassified - Hypothetical Protein Supported by cDNA	3.18
Gma.17498.1.S1_at	Hypothetical protein T22E16.190	Unclassified - Protein with Unknown Function	1.3 C

***** Genes also identified as up-regulated in Conrad are indicated by "C"

Those differentially expressed genes up-regulated in Sloan at day 2 post inoculation (Table 4.5) include two putative genes in the 'Metabolism' functional category, Digalactosyldiacylglycerol (DGDG) synthase 1, in a recent study, the importance of auxin transport for accumunlation of DGDG during phosphate (Pi) starvation was discussed. The glycolipid plays an important role in chloroplast function and makes up the bulk of membrane lipids (Narise et al., 2010).

Table 4.6. Functional classification of genes significantly up-regulated exclusively at 2 dpi in V71-370.

Gene ID	Annotation	Functional Categories	Infection response (fold change) *
Gma.2055.1.S1_s_at	SNARE domain containing protein, expressed	Cell Growth & Division	2.51
GmaAffx.87547.2.S1_at	Glycoside hydrolase, family 1	Cell Structure	6.7
Gma.8731.1.S1_at	Hypothetical protein F14P22.80	Metabolism	2.23
GmaAffx.37270.1.S1_at	Function unknown	No Homology to Known Proteins	1.66

GmaAffx.41649.1.S1_at	Function unknown	No Homology to Known Proteins	6.98
GmaAffx.69402.1.S1_at	Expressed protein	No Homology to Known Proteins	6.13
Gma.7789.2.S1_s_at	Ubiquitin C variant	Protein Destination & Storage	1.29 C
Gma.4868.1.A1_at	Function unknown	Signal Transduction	3.3
Gma.6708.1.A1_at	Protein kinase	Signal Transduction	2.03
Gma.16640.2.S1_at	Ttg1-like protein	Transcription	2.68
GmaAffx.84921.2.S1_at	Function unknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.45
Gma.13076.1.S1_at	Hypothetical protein P0636E04.6	Unclassified - Hypothetical Protein Supported by cDNA	4.68
GmaAffx.28655.1.S1_at	Arabidopsis thaliana genomic DNA, chromosome 3, BAC clone:T5M7	Unclassified - Hypothetical Protein Supported by cDNA	4.36
GmaAffx.93318.1.S1_s_at	Hypothetical protein P0636E04.6	Unclassified - Hypothetical Protein Supported by cDNA	5.09
Gma.15359.1.S1_x_at	Similarity to GTPase activating protein	Unclassified - Protein with Unknown Function	1.47

***** Genes also identified as up-regulated in Conrad are indicated by "C"

Among those transcripts in V71-370 specifically up-regulated at 2 dpi (Table 4.6), many are classified within the 'Signal Transduction'' functional category, though many are also of unknown function. For example, Gma.6708.1.A1_at, annotated as a Protein Kinase. Protein kinases are known to play key roles in many aspects of plant defense signaling. (Romeis et al., 2001)The cascades of mitogen-activated protein kinase (MAPK) in eukaryotic cells act as important signaling modules. For example, the activation of a Tobacco mitogen-activated protein kinase (Ntf4) during plant defense response and it had a cell like death similar to a hypersensitive response (HR). The cryptogenin which accelerates a HR cell like death in tobacco was also shown to accelerate a HR response in plants treated with Ntf4 (Ren et al., 2006).

Table 4.7. Functional classification of genes significantly up-regulated exclusively at 2 dpi in VPRIL9

Gene ID	**Annotation**	**Functional Categories**	**Infection response (fold change) ***
Gma.16707.1.S1_a_at	Function unknown	Disease & Defense	2.58
GmaAffx.84858.1.S1_at	Disease resistance protein; AAA ATPase	Disease & Defense	1.34
GmaAffx.92922.1.S1_s_at	TIR	Disease & Defense	2.38

Gma.10666.1.S1_at	Alcohol dehydrogenase	Metabolism	1.38
GmaAffx.36823.1.S1_at	Function unknown	No Homology to Known Proteins	1.91
GmaAffx.53749.1.A1_at	Function unknown	No Homology to Known Proteins	1.68
GmaAffx.93596.1.S1_at	Function unknown	No Homology to Known Proteins	1.79
Gma.3473.1.S1_at	Function unknown	Protein Destination & Storage	7.75 C
Gma.1677.1.S1_a_at	DSBA oxidoreductase	Secondary Metabolism	1.35
GmaAffx.81051.2.S1_at	similar to protein kinase AtSIK	Signal Transduction	2.04
Gma.16547.1.S1_at	WRKY86	Transcription	1.8
Gma.8372.2.S1_at	Dehydration-responsive element binding protein 2	Transcription	2.95
GmaAffx.86377.1.S1_at	Putative WRKY transcription factor 30	Transcription	3.38
Gma.8221.1.A1_at	Hypothetical protein At3g45040	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.42
GmaAffx.48332.1.S1_at	Arabidopsis thaliana genomic DNA, chromosome 3, BAC clone:F5N5	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.6
Gma.1730.1.A1_at	Gb|AAF02142.1	Unclassified - Hypothetical Protein Supported by cDNA	1.7
Gma.17802.1.S1_at	UVI1	Unclassified - Hypothetical Protein Supported by cDNA	5.35

***** Genes also identified as up-regulated in Conrad are indicated by "C"

Among those transcripts in VPRIL-9 specifically up-regulated at 2 dpi (Table 4.7), the functional groups with the highest number of differentially expressed transcripts were 'Disease and Defense' and 'Transcription' each equally containing three transcripts. Two of the latter classes are annotated as WRKY transcription factors. WRKY transcription factors have been widely studied in different pathosystems including Arabidopsis pathosystems where they are crucial regulators of the defense transcriptome and disease resistance (Eulgem and Somssich, 2007). Within 'Disease and Defense' GmaAffx.84858.1.S1_at is annotated as an AAA ATPase. These enzymes play wide roles in cellular processes required for plant development and growth such as ubiquitin-dependent processes, membrane trafficking and fusion, protein folding and proteolysis(Hicks-Berger et al., 2006).

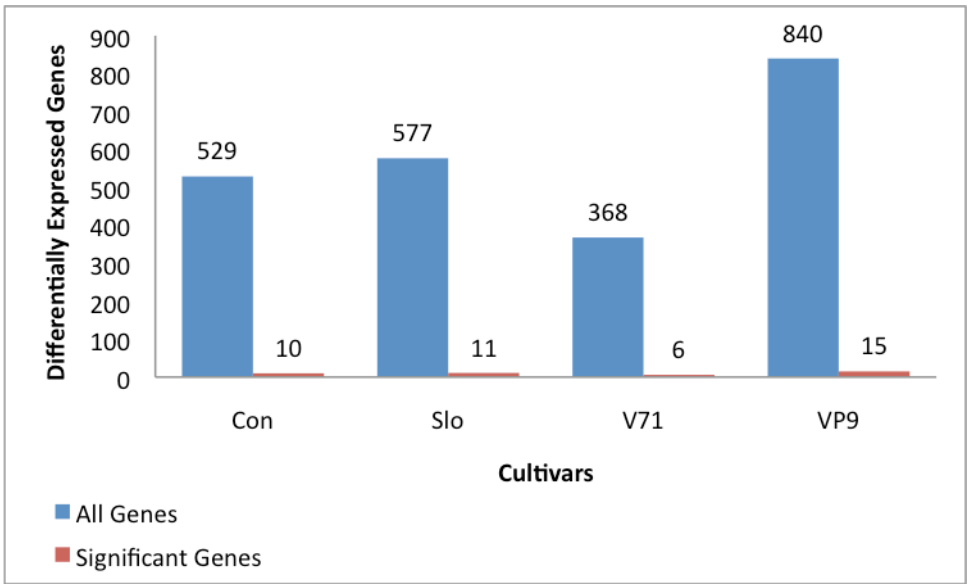


Figure 4.5. Differentially expressed genes up-regulated exclusively at 3 days post inoculation. Resistant lines: V71-370 (V71) and Conrad (Con). Susceptible lines Sloan (Slo) and VPRIL-9 (VP9).

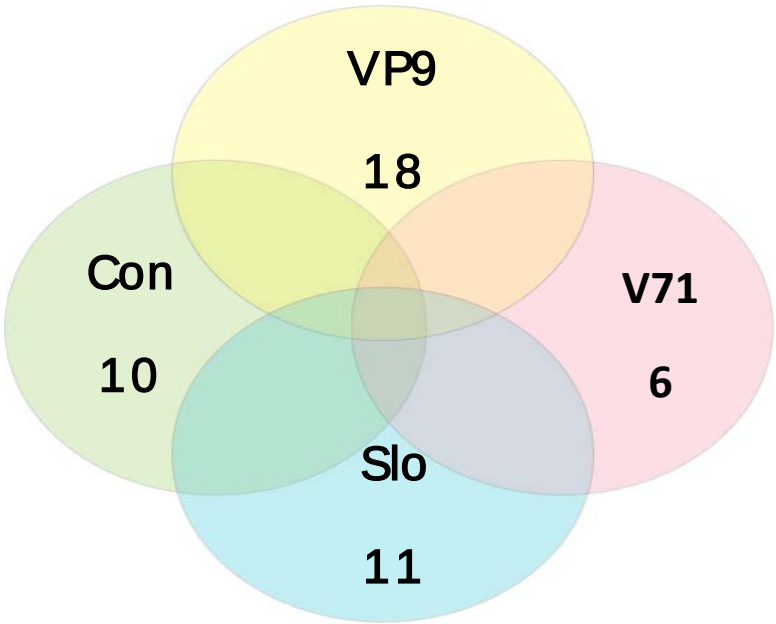


Figure 4.6 Venn diagram of shared differentially expressed cultivars exclusively at 3 day post inoculation Resistant lines: V71-370 (V71) and Conrad (Con). Susceptible lines Sloan (Slo) and VPRIL-9 (VP9). There were no differentially expressed genes shared among cultivars during 3dpi.

Among the four cultivars, less than two percent of the differentially expressed genes were found to be significantly up-regulated at day 3 but not at day 1, day 2, or day 5 post-inoculation. Fifteen genes were significantly up-regulated in the susceptible cultivar VPRIL-9 and many of those were in the functional groups 'Metabolism' and 'Protein Destination and Storage'. Two MYB Transcription factors were unique to the most resistant cultivar V71-370.

Table 4.8. Functional classification of genes significantly up-regulated at 3 dpi in each cultivar.

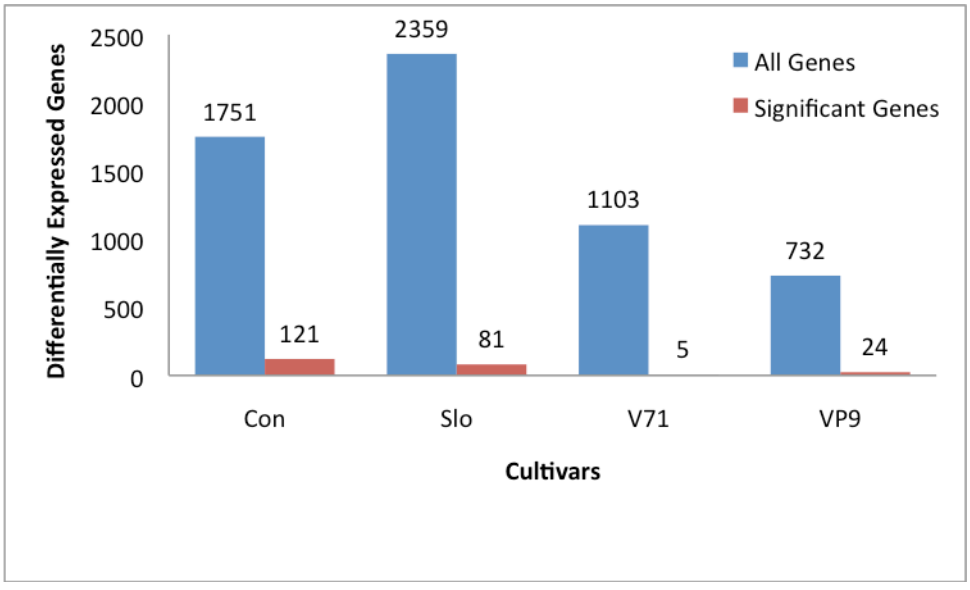
Gene ID	Annotation	Functional Categories	Infection response (fold change)
Conrad (resistant)			
Gma.7497.1.S1_a_at	Pyridoxal 5 phosphate dependent enzyme, beta subunit	Metabolism	1.26
GmaAffx.82240.1.S1_at	UDP-glucuronosyl/UDP-glucosyltransferase	Metabolism	2.72
Gma.4721.1.A1_s_at	Hypothetical protein	No Homology to Known Proteins	1.44
Gma.7504.2.S1_a_at	Ribosomal protein L14b/L23e	Protein Synthesis	1.38
GmaAffx.44071.2.S1_at	Putative ras GTPase activating protein SH3 domain binding protein	Signal Transduction	1.34
GmaAffx.58953.1.S1_at	Hypothetical protein OJ2056 H01.18	Unclassified - Hypothetical Protein Supported by cDNA	1.4
GmaAffx.63601.1.S1_at	AT5g50310/MXI22 1	Unclassified - Hypothetical Protein Supported by cDNA	1.53
GmaAffx.92577.1.S1_s_at	Adenosine kinase	Unclassified - Hypothetical Protein Supported by cDNA	1.75
Gma.12886.1.A1_at	Protein kinase-like	Unclassified - Protein with Unknown Function	1.81
Gma.5214.1.S1_at	Hypothetical protein F9D16.100	Unclassified - Protein with Unknown Function	1.52
V71-370 (resistant)			
Gma.7328.2.S1_at	Pyridoxal kinase	Metabolism	1.71
GmaAffx.67202.1.S1_at	Cycloartenol synthase	Secondary Metabolism	1.42
Gma.18049.2.S1_at	MYB transcription factor MYB84	Transcription	1.59
Gma.8189.1.A1_at	MYB transcription factor MYB102	Transcription	2.7
GmaAffx.42439.1.S1_at	Transposon protein	Transposon	2.03
Gma.7956.1.A1_at	Metal tolerance protein C2	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.44
Sloan (susceptible)			
Gma.13160.1.S1_at	Cytosolic ascorbate peroxidase 1	Disease & Defense	1.17
Gma.5018.1.S1_at	ATP citrate lyase b-subunit	Metabolism	2.01

Gma.15554.2.A1_at	T21P5.16 protein	No Homology to Known Proteins	1.54
GmaAffx.50536.1.A1_s_at	DNA-binding WRKY	No Homology to Known Proteins	2.06
Gma.16753.2.S1_a_at	ATP-dependent Clp protease proteolytic subunit	Protein Destination & Storage	1.54
GmaAffx.87100.1.S1_s_at	Isoleucine-tRNA ligase-like protein	Protein Synthesis	1.36
Gma.10043.1.A1_at	Adenine nucleotide translocator 1	Transporter	1.53
GmaAffx.65347.1.S1_at	Hypothetical protein	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.46
GmaAffx.66348.2.S1_at	Function unknown	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.31
GmaAffx.23.2.S1_at	Hypothetical protein T16K5.70	Unclassified - Hypothetical Protein Supported by cDNA	1.73
GmaAffx.60423.1.S1_at	SAM_(And_some_other_nucleotide) binding motif; Skb1 methyltransferase	Unclassified - Protein with Unknown Function	1.38
VPRIL9 (susceptible)			
GmaAffx.91997.1.S1_at	Similarity to ADP-ribosylation factor	Intracellular Traffic	1.32
Gma.6602.1.S1_at	Function unknown	Metabolism	2.32
GmaAffx.11053.1.A1_at	OSJNBa0084K01.2 protein	Metabolism	1.99
GmaAffx.35772.1.S1_x_at	Cytochrome P450 monooxygenase CYP710A15	Metabolism	2.14
GmaAffx.21514.1.A1_at	Function unknown	No Homology to Known Proteins	1.92
GmaAffx.91988.1.S1_at	Function unknown	No Homology to Known Proteins	1.57
Gma.11179.1.S1_s_at	DnaK protein	Protein Destination & Storage	1.57
Gma.1751.1.S1_at	Mitochondrial processing peptidase beta subunit	Protein Destination & Storage	1.72
GmaAffx.27477.1.S1_at	F20D23.20 protein Arabidopsis thaliana related	Protein Destination & Storage	1.51
GmaAffx.92139.1.S1_x_at	Subtilisin-like protease	Protein Destination & Storage	2.63
GmaAffx.53996.1.A1_at	Protein At2g44750	Secondary Metabolism	1.89
GmaAffx.92880.1.S1_s_at	Mitochrondrial voltage dependent anion selective channel	Transporter	1.31
GmaAffx.71827.1.S1_at	Hypothetical protein At2g01410	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.23
GmaAffx.86216.1.S1_at	Secondary cell wall-related glycosyltransferase family 47	Unclassified - Hypothetical Protein NOT Supported by cDNA	1.56
GmaAffx.84575.1.S1_at	Arabidopsis thaliana genomic DNA, chromosome 5, TAC clone:K17O22	Unclassified - Hypothetical Protein Supported by cDNA	1.54

The functional category with the highest number of significantly up-regulated genes was ‘Metabolism’ with 6 up-regulated. The functional group ‘Protein Destination’ was unique to those differentially expressed genes in the two susceptible cultivars. Sloan also contained a

unique differentially expressed gene in 'Disease and Defense' Gma.13160.1.S1_at **(**Cytosolic ascorbate peroxidase 1)**.** *Arabidopsis* demonstrated complete scavenging system collapse in the absence of cytosolic ascorbate peroxidase 1, allowing protein oxidation to occur because H_2O_2 levels increase (Davletova et al., 2005).

Figure 4.7. Differentially expressed genes up-regulated exclusively at 5 days post inoculation



Resistant lines: V71-370 (V71) and Conrad (Con). Susceptible lines Sloan (Slo) and VPRIL-9 (VP9).

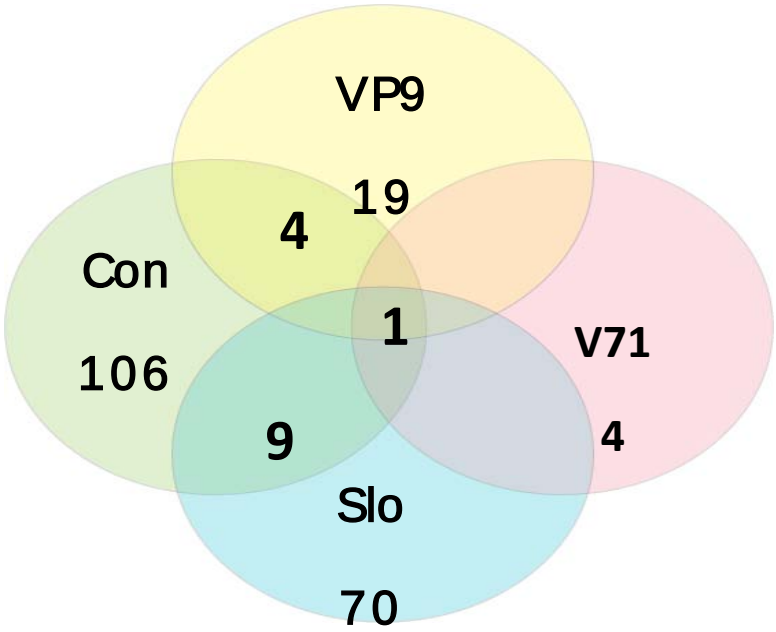


Figure 4.8 Venn diagram of shared differentially expressed cultivars exclusively at 5 day post inoculation Resistant cultivars (Con and V71) and susceptible cultivars (VP9 and Slo). The number of differentially expressed genes shared between each cultivar. There were more differentially expressed genes shared among with all cultivars during 5 dpi.

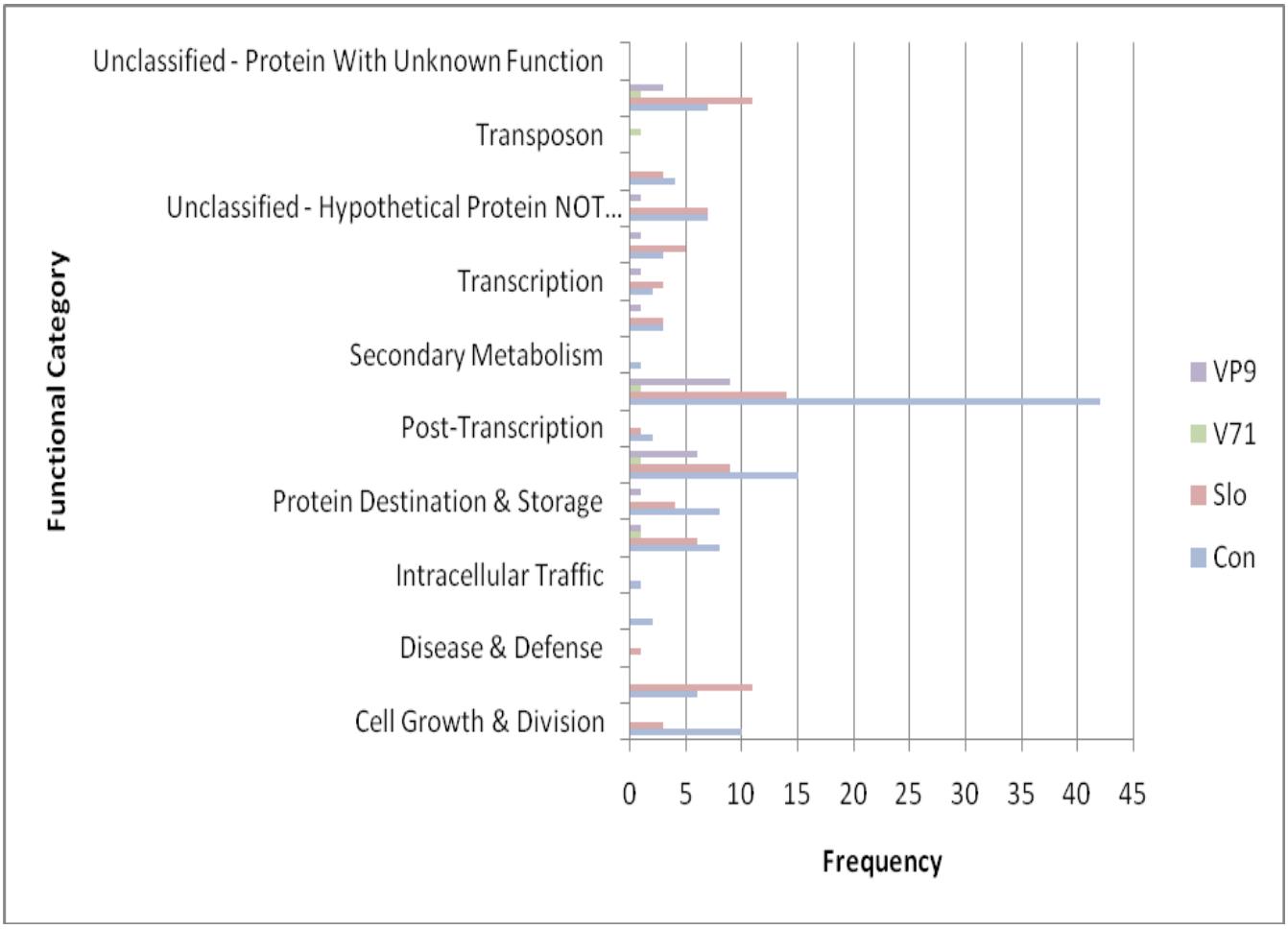


Figure 4.9. Distribution of functional categories among genes up-regulated specifically at day 5.

The number of transcripts up-regulated exclusively at day 5 was highly variable, even among resistant or among susceptible cultivars (Figure 4.7). There were only five (0.45%) significantly up-regulated in V71-370 specific to day 5. The most highly represented functional category among all cultivars was 'Protein Synthesis' (Figure 4.9). Conrad had a total of 121 significantly up-regulated genes exclusive to day 5 with 34.7% of those genes identified in the functional category 'Protein Synthesis' while Sloan (17.3%), V71-370 (20%) and VPRIL-9 (38%) also displayed large numbers of genes in this functional category.

Figure 4.10. Differentially expressed genes significant up-regulated at 1 day and 2 days post inoculation

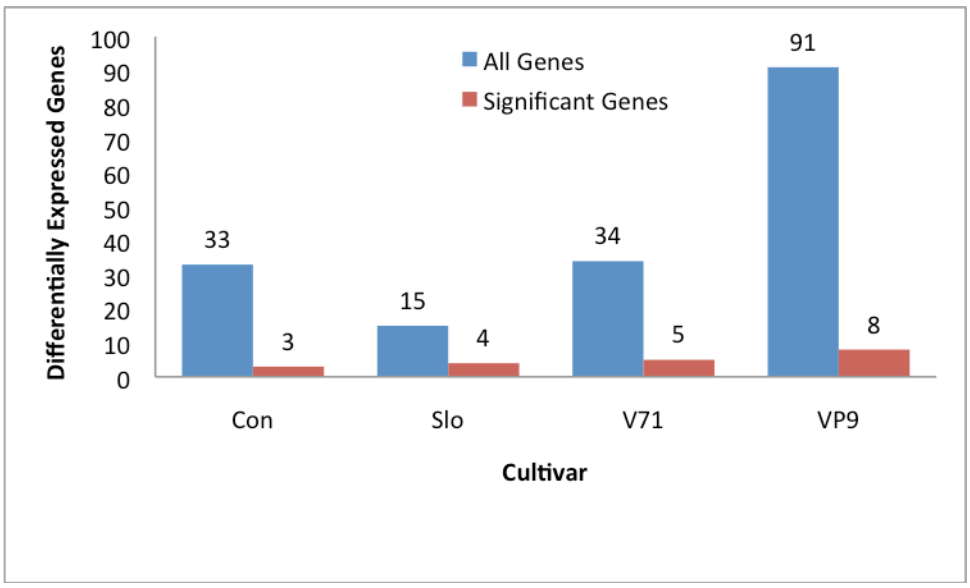
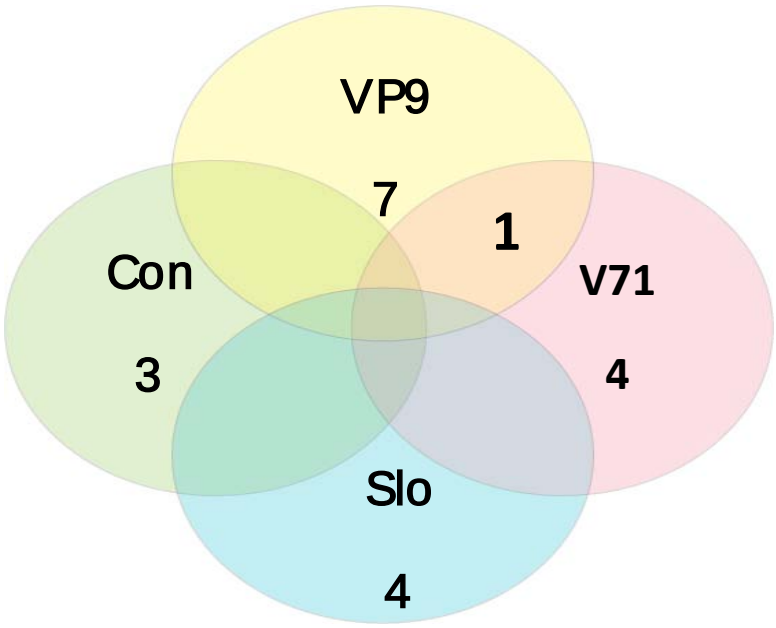


Figure 4.11 Venn diagram of shared differentially expressed cultivars exclusively at 1 and 2 day post inoculation



Resistant cultivars (Con and V71) and susceptible cultivars (VP9 and Slo). The number of differentially expressed genes shared between each cultivar. There were more differentially expressed genes shared among with all cultivars during 1 and 2 dpi.

Table 4.9. Transcripts significantly up-regulated at both day 1 and day 2 post inoculation in each cultivar, but not at days 3 or 5.

Gene ID	Annotation	Functional Categories	Infection response (fold change)
Conrad (resistant)			
GmaAffx.10164.1.A1_at	Function unknown	No Homology to Known Proteins	2.71
Gma.13518.1.S1_at	Amino acid transporter-like protein	Transporter	1.65
Gma.4460.1.S1_at	Inositol polyphosphate kinase	Unclassified - Hypothetical Protein Supported by cDNA	1.86
V71-370 (resistant)			
Gma.9560.1.A1_at	Function unknown	Disease & Defense	3.59
Gma.5258.1.S1_at	Function unknown	No Homology to Known Proteins	1.87
GmaAffx.43947.1.S1_at	Function unknown	Signal Transduction	3.06
GmaAffx.78968.1.S1_at	Calcium-binding EF-hand	Signal Transduction	3.92
GmaAffx.36938.1.S1_at	WRKY DNA-binding protein	Transcription	7.03
GmaAffx.37095.2.S1_x_at	Functional candidate resistance protein KR1	Disease & Defense	3.58
Gma.58.1.S1_at	RING-H2 finger protein	Protein Destination & Storage	1.87
GmaAffx.74458.1.A1_at	Serine/threonine kinase-like protein	Signal Transduction	3.05
Sloan (susceptible)			
Gma.10818.3.S1_at	WRKY78	Transcription	2.17
Gma.2655.2.S1_a_at	Prefoldin; Helix-loop-helix DNA-binding	Transcription	1.24
GmaAffx.87526.1.S1_at	Function unknown	Unclassified - Hypothetical Protein Supported by cDNA	5.98
Gma.14802.1.S1_at	Emb|CAB70981.1	Unclassified - Protein with Unknown Function	4.82
VPRIL9 (susceptible)			
GmaAffx.37095.2.S1_x_at	Functional candidate resistance protein KR1	Disease & Defense	4.41
Gma.58.1.S1_at	RING-H2 finger protein	Protein Destination & Storage	1.88
GmaAffx.74458.1.A1_at	Serine/threonine kinase-like protein	Signal Transduction	2.19
GmaAffx.37095.2.S1_x_at	Functional candidate resistance protein KR1	Disease & Defense	4.35
Gma.58.1.S1_at	RING-H2 finger protein	Protein Destination & Storage	9.58
GmaAffx.74458.1.A1_at	Serine/threonine kinase-like protein	Signal Transduction	2.41
GmaAffx.86392.1.S1_at	Heavy metal-induced protein 6A	Signal Transduction	1.53
GmaAffx.36938.1.S1_at	WRKY DNA-binding protein	Transcription	9.55
GmaAffx.9653.1.S1_at	Zinc finger-like protein	Transcription	2.41

Gma.8388.1.S1_at	F16F4.11 protein	Unclassified - Hypothetical Protein Supported by cDNA	1.52
GmaAffx.5200.1.S1_at	Arabidopsis thaliana genomic DNA, chromosome 5, TAC clone:K17O22	Unclassified - Hypothetical Protein Supported by cDNA	1.51

There were few transcripts significantly up-regulated at both 1 dpi and 2 dpi, but not 3 dpi or 5 dpi (Table 4.9). The least number of significant putative genes differentially expressed was identified in Conrad, Transcription factors were shown to be significantly differentially expressed among susceptible cultivars Sloan and VPRIL-9.

Figure 4.12. Transcripts significantly up-regulated at both day 3 and day 5 post inoculation in each cultivar, but not at days 1 or 2.

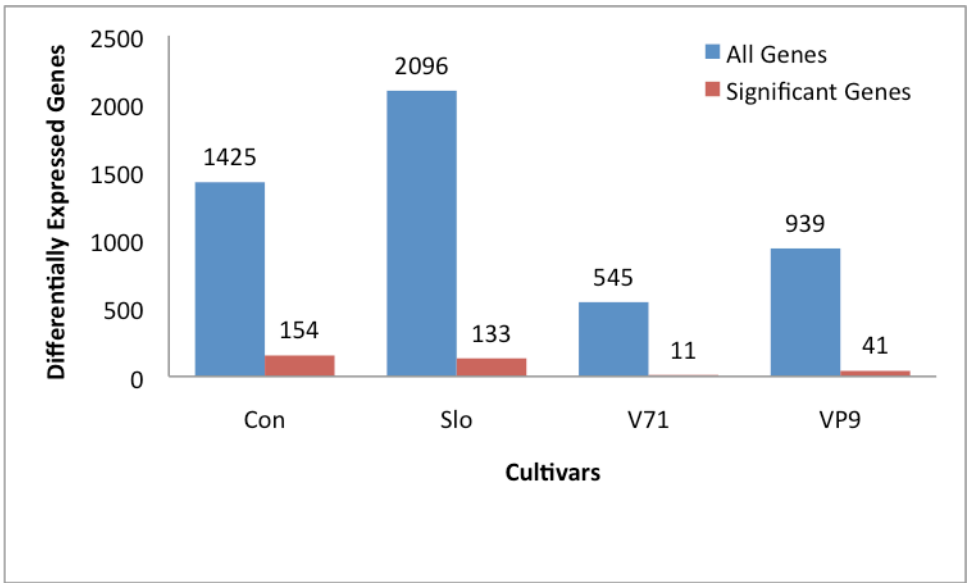
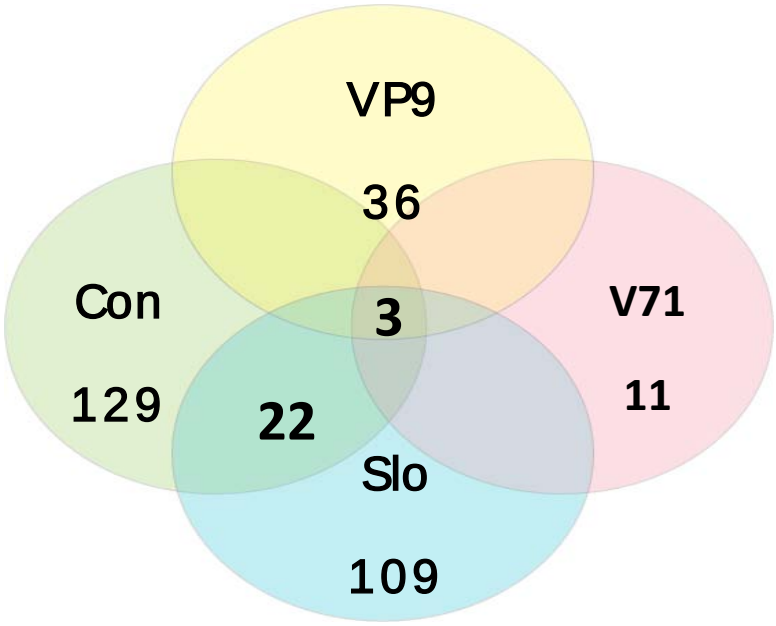


Figure 4.13 Venn diagram of shared differentially expressed cultivars exclusively at 3 and 5 day post inoculation



Resistant cultivars (Con and V71) and susceptible cultivars (VP9 and Slo). The number of differentially expressed genes shared between each cultivar. There were more differentially expressed genes shared among Conrad and both susceptible cultivar during 3 and 5 dpi.

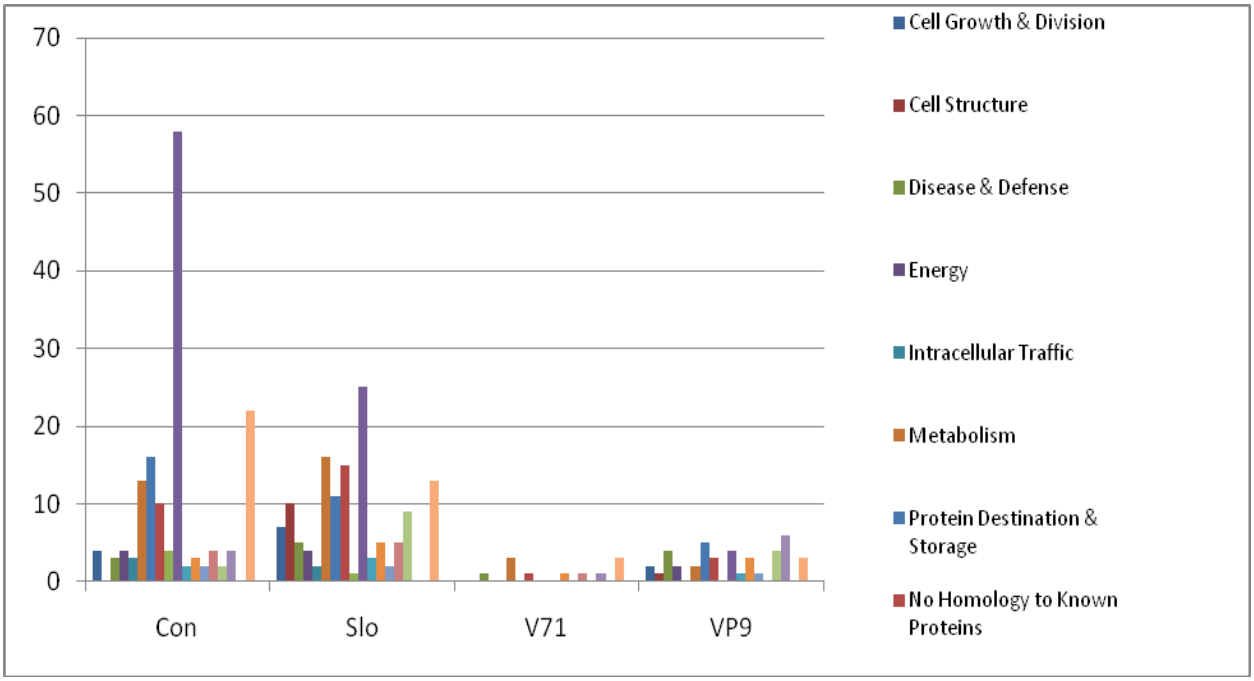


Figure 4.14. Distribution of functional categories among genes up-regulated specifically at day 3 and 5.

Many more transcripts were significantly up-regulated at days 3 and 5 than at days 1 and 2 (Figure 4.12), as expected due to the expansion of the infection over time. This trend was true for every cultivar individually, but was especially large in the case of Conrad and Sloan. In Conrad 38% of the transcripts up-regulated at both days 3 and 5 were identified in the functional category 'Protein Synthesis' which includes many ribosomal proteins and elongation factor proteins. The resistant cultivars displayed at least 40% more up-regulated transcripts involved in 'Protein Synthesis' compared to the susceptible cultivars, while the susceptible cultivars identified more up-regulated transcripts involved in 'Metabolism' and 'Disease and Defense'. A putative Histone deacetylase 2a was identified in the 'Transcription' functional category. Histone deacetylases are associated with repressing transcription, specifically those plants were identified as more resistant if they over express HDA19 (Milner, 2008). Many defense genes are regulated by WRKY transcription factors(Eulgem and Somssich, 2007) and in plant basal defense of Arabidopsis, TFs WRKY38 and WRKY62 interact with Histone deacetylase

(HDA19) (Milner, 2008). HDA19 was identified as a global repressor that in involved in many basal defense responses such as development, stress responses, ethylene signaling, and jasmonic acid (Hollender and Liu, 2008).

In this study to identify significant differentially expressed genes of four cultivars varying in resistance, the groups of unknown genes are also interesting because when the functions are identified they may produce new findings relating to partial resistance.

In our study we focused on defense response, in four cultivars with varying levels of resistance, over time. During the early time points 1 and 2 day post inoculation many categories of transcripts were associated with transcription, signal transduction and Protein Destination and Storage. These genes varied with the different genotypes of different levels of quantitative resistance. Futhermore, our results indicate that each genotype may have its own unique set of genes contributing to quantitative resistance.

Our findings, are consistent with a recent soybean transcriptome study, that the majority of the transcriptional differences are less than two-fold in magnitude (Zhou et al., 2009). The soybean transcriptome study also suggests the less than two-fold changes are highly statistically significant (Zhou et al., 2009). These genes may be involved in quantitative resistance and represent an excellent resource for future studies of plant defense mechanisms. Although many genes in our study are not expressed during early time points our findings suggest the small number of genes displaying differences among days may reflect those genes unusually sensitive over time.

In an infection time course study by Moy et al. (Moy et al., 2004) changes in gene expression were tracked during much early time points, which may contribute to the small number of genes identified during our study. The early time point of our experiment 24 hours was identified by Moy et al as the infection process turning point. Our findings showed many of the genes differentially expressed took place in resistant cultivar Conrad during those early time points 1 dpi or 24 hours which may identify genes in quantitative resistance.

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Chapter 5

Differentially expressed genes potentially involved in regulation during early time points of infection

5.1 Introduction

Plants respond to pathogens by means of a network of signaling pathways that stimulate transcriptional activation of defense related genes. To understand the molecular and cellular mechanisms underlying host responses to pathogen challenge in soybean, we focused on potential regulatory genes that are differentially expressed in four soybean cultivars in response to infection by *P. sojae.*

Identifying potential transcription factors (TFs) that are up-or down-regulated early in infection may help us to discover TFs that control the earliest responses to infection and/or that regulate quantitative resistance (QR). QR is controlled by multiple genes and has been shown to be a durable form of resistance to pathogens affecting cultivated crops including soybean (Buzzell and Anderson, 1982). Not many known genes underlying QR have been identified (Kou and Wang). Understanding which genes are expressed during early time points of infection will aid in unraveling the complex processes of the soybean infection response and in identification of the mechanisms involved. Global transcriptional profiling utilizing Affymetrix microarray technology was used to identify genes that were differentially expressed in four cultivars with varying levels of field resistance to *P. sojae.* Profiles at different time points following inoculation were compared to identify regulatory factors that may contribute to defense. Selected genes were then mapped to soybean molecular linkage maps. These efforts also add to the current linkage maps which can serve as a resource for breeders in discovering novel genes conferring resistance to *P. sojae* in the field.

5.2 Objective

Identify genes that may have a regulatory function that are differentially expressed during the earliest time points of infection and map them onto current soybean molecular linkage groups. These genes may potentially contribute to quantitative resistance.

5.3 Results

5.3.1 Identification of putative soybean regulatory genes differentially expressed in resistant compared to susceptible cultivars

As described in Chapter 4, we examined global gene expression in soybean cultivars Conrad, V71-370, VPRIL-9 (VP9) and Sloan at 1, 2, 3 and 5 days post inoculation (dpi) with *P. sojae*. This resulted in the identification of 30,166 genes with detectable transcripts. To identify potential regulatory genes associated with partial resistance to *P. sojae,* we identified genes that had statistically significant variation in expression among the different cultivars at each time point. Linear Mixed Model Analysis of Variance (LMMA) was used, and the false discovery rate was controlled at the 0.05 level using the TST-FDR method (Benjamini et al., 2006) The Response Contrast (ratio of pathogen-inoculated to mock-inoculated contrasted between two cultivars) was used to look for genes with expression patterns common to the most resistant cultivars (Conrad and V71-370) in contrast to the susceptible reference cultivars (Sloan and RIL-9) during early infection (1 dpi and 2 dpi). Genes putatively associated with transcriptional regulation were selected for further investigation.

Table 5.1 Transcription-related genes with a positive response contrast in a comparison of resistant and susceptible lines

Contrast	1 dpi		2 dpi	
	All*	Significant†	All	Significant
V71-370 (R) vs VPRIL-9 (S)	7	1	8	1
V71-370 (R) vs Sloan (S)	5	0	34	6
Conrad (R) vs VPRIL-9 (S)	5	0	37	7
Conrad (R) vs Sloan (S)	3	1	6	3

*Represents all transcription-associated genes that have a positive response contrast for the indicated comparisons

† Significant at the 0.05 level following TST-FDR control.

Table 5.1 displays a total of 105 genes with a positive response contrast between resistant and susceptible lines at 1 dpi and 2 dpi. To identify those genes that might be associated with QR, the "Infection Responses" (ratio of pathogen-inoculated to mock-inoculated) of the four cultivars were compared among each other. Altogether four cultivar contrasts were used to characterize interactions among each of the genotypes (listed in column 1 of Table 5.1). For all the cultivars, fewer genes showed positive response contrasts at 1 dpi compared to 2 dpi, with a similar trend observed for the number of genes significantly differentially expressed at 1 dpi compared to 2 dpi. Of the 19 genes putatively associated with transcriptional regulation, all were significantly differentially induced when comparing resistant versus susceptible lines, two were identified at 1 dpi and 17 at 2 dpi. Interestingly the genes identified at 1 dpi did not overlap with those identified at 2 dpi. Amongst the four cultivars, the contrast between the resistant cultivar Conrad and the susceptible line VPRIL-9 identified the greatest number of putative regulatory genes followed closely by the contrast between V71-370 (resistant) and Sloan (susceptible). Additionally, there were no gene overlaps between the two contrasts. Notable among these genes are members of the extensively studied MYB, WRKY and the NAC domain-containing transcription factors. In most cases, the putative regulatory genes were up-regulated in the resistant cultivar. For example, for the Conrad versus VPRIL9 comparison, the CONSTANS-like putative zinc finger protein transcript and the two NAC domain protein transcripts (which derive from homeologous genes) are strongly differentially up-regulated.

Table 5.2: Potential soybean regulatory genes differentially expressed at 1dpi

			Infection responses*		
Contrast	**Gene ID**	**Annotations**	**R**	**S**	**Contrast**
V71 vs VP9	GmaAffx.76123.1.S1_at	LOB domain containing protein 3	-1.0	-1.66 †	1.66
Con vs Slo	GmaAffx.5966.1.A1_at	Hypothetical protein SHOOT2	1.22	-1.33	1.62

* Infection responses in the resistant (R) cultivar (V71-370 or Conrad) and in the susceptible (S) cultivars (Sloan or VPRIL9)

† significant infection response at the 0.05 level following TST-FDR control

Table 5.3: Potential soybean regulatory genes differentially expressed at 2 dpi

Contrast	Gene ID	Annotations	Infection Response*		Contrast
			R	S	
V71 vs VP9	GmaAffx.20956.1.S1_at	MYB transcription factor MYB118	1.16	-1.7 †	1.5
Con vs Slo	Gma.4014.2.S1_at	Transcription factor BIM1	1.07	-2.25 †	2.4
Con vs Slo	GmaAffx.12933.1.S1_at	Nuclear transcription factor Y subunit A-1	1.02	-1.11 †	1.1
Con vs Slo	GmaAffx.78736.1.S1_at	Histone-lysineN-	1.41	-1.22	1.7
V71 vs Slo	Gma.18069.1.S1_at	WRKY54	-1.18	-1.78 †	1.5
V71 vs Slo	GmaAffx.1301.20.S1_at	At1g10120	-1.47	-2.9 †	2.0
V71 vs Slo	Gma.11276.1.S1_at	Similarity to Myb	-1.22	-2.4 †	2.0
V71 vs Slo	GmaAffx.83081.1.S1_at	Histone deacetylase superfamily	1.19	-1.5 †	1.8
V71 vs Slo	GmaAffx.82726.1.S1_at	Hypothetical protein At3g21300	-1.34	-3.1 †	2.3
V71 vs Slo	GmaAffx.85063.1.S1_at	Class III HD-Zip protein 1	-1.14	-1.95 †	1.7
Con vs VP9	Gma.1649.1.A1_at	NAC domain protein NAC3	16.9 †	2.4	7.0
Con vs VP9	GmaAffx.38168.1.S1_at	SNF2P	1.07	-1.36 †	1.5
Con vs VP9	Gma.6380.1.S1_at	Ethylene-responsive element	2.36 †	1.37 †	1.7
Con vs VP9	GmaAffx.40296.1.A1_at	Zinc finger protein CONSTANS-LIKE 15	12.3 †	2.68 †	4.6
Con vs VP9	GmaAffx.43744.1.S1_at	Pollen specific LIM domain protein 1a	-1.07	-2.16 †	2.0
Con vs VP9	GmaAffx.57970.2.S1_at	NAC domain protein NAC3	17.4 †	2.84	6.1
Con vs VP9	GmaAffx.86401.1.S1_at	SBP	2.2 †	1.25	1.8

* Infection responses in the resistant (R) cultivar (V71-370 or Conrad) and in the susceptible (S) cultivars (Sloan or VPRIL9)

† significant infection response at the 0.05 level following TST-FDR control

5.3.2 Mapping regulatory genes to soybean molecular linkage groups

Putative regulatory genes with a positive response contrast (TST FDR < 0.05) between at least one pair of resistance and susceptible cultivars were selected for genetic mapping. Two transcripts identified from day 1 and seventeen identified from day 2 were mapped. The map

positions of the genes encoding the transcripts were compared to those of known phenotypic QTL that control *P. sojae* resistance in soybean. Four QTL were mapped in the cross V71-370 x PI407162, on Marker Linkage Groups (MLG)-G (10-35 cM), I (25-50 cM), J (65-95 cM) and D1A (40-70 cM) (Tucker et al., 2010) while six were mapped in the cross Conrad x Sloan, on MLG-H (37-63 cM), F (16-24 cM and 21-32 cM), B2 (23-32 cM), D2 (25-40 cM) and L (33-37 cM) (Wang et al., 2010). The map positions of the genes were determined by matching the transcript sequence to the genome sequence and then matching that portion of the genome sequence to a high density Single Feature Polymorphism (SFP) map of soybean (Krampis, 2009). The putative regulatory genes mapped to at least one soybean linkage group (Table 5.4).

Table 5.4 Map locations of transcripts with significant response contrasts

Contrast	Gene ID	Annotations	Contrast (fold)	MLG	Position (cM)
Day 1					
V71 vs VP9	GmaAffx.76123.1.S1_at	LOB domain containing protein 3	1.66	A2, E	70, 35
Con vs Slo	GmaAffx.5966.1.A1_at	Hypothetical protein SHOOT2	1.62	J	90*
Day 2					
V71 vs VP9	GmaAffx.20956.1.S1_at	MYB transcription factor MYB118	1.5	O	55
Con vs Slo	Gma.4014.2.S1_at	Transcription factor BIM1	2.4	A1	20
Con vs Slo	GmaAffx.12933.1.S1_at	Nuclear transcription factor Y subunit A-1	1.1	G	50
Con vs Slo	GmaAffx.78736.1.S1_at	Histone-lysineN- methyltransferase SUVR5	1.7	D1a	115
V71 vs Slo	Gma.18069.1.S1_at	WRKY54	1.5	D1b	23
V71 vs Slo	GmaAffx.1301.20.S1_at	At1g10120	2.0	B2	50
V71 vs Slo	Gma.11276.1.S1_at	Similarity to Myb	2.0	D1B	115
V71 vs Slo	GmaAffx.83081.1.S1_at	Histone deacetylase superfamily	1.8	C1	74
V71 vs Slo	GmaAffx.82726.1.S1_at	Hypothetical protein At3g21300	2.3	F	115
V71 vs Slo	GmaAffx.85063.1.S1_at	Class III HD-Zip protein 1	1.7	L	25†
Con vs VP9	Gma.1649.1.A1_at ‡	NAC domain protein NAC3	7.0	I	15
Con vs VP9	GmaAffx.38168.1.S1_at	SNF2P	1.5	F	60
Con vs VP9	Gma.6380.1.S1_at	Ethylene-responsive element	1.7	B2	65
Con vs VP9	GmaAffx.40296.1.A1_at	Zinc finger protein CONSTANS-LIKE 15	4.6	L	40†
Con vs VP9	GmaAffx.43744.1.S1_at	Pollen specific LIM domain protein 1a	2.0	B2	50
Con vs VP9	GmaAffx.57970.2.S1_at ‡	NAC domain protein GmNAC3	6.1	C2	110
Con vs VP9	GmaAffx.86401.1.S1_at	SBP	1.8	M	75

* falls within QTL mapped in V71-370 x PI407162 at 65-95 cM

† falls close to a QTL mapped in Conrad x Sloan at 33-37 cM

‡ these two transcripts derive from homeologous genes

5.4 Discussion

Identifying regulatory factors differentially expressed in resistant cultivars may provide insights into the regulatory mechanisms underlying partial (or quantitative) resistance to *P. sojae*. The recently sequenced soybean genome identified a total of 5,671 putative soybean transcription factor genes, distributed in 63 families, which represents 12.2% of the 46,430 predicted soybean protein-coding genes (Schmutz et al., 2010). In this study we observed very few regulatory genes, specifically transcription factors, differentially expressed when comparing the resistant and susceptible cultivars. This observation may have biological significance. Alternatively, as Zhou and associates (Zhou et al., 2009) pointed out, there may have been some relatively low magnitude changes which were masked by technical and biological variability, given the relatively low number of replications (four). Amongst the four cultivars, the comparison between the resistant cultivar Conrad and the susceptible line VPRIL9 identified the most putative regulatory genes, followed closely by the contrast between V71-370 (resistant) and Sloan (susceptible). Additionally there were no gene overlaps between the two pairs of contrasts, suggesting that regulation of defense-related genes in these cultivars is controlled by different transcription factors. Alternatively, if the experiment had comparatively weak statistical power to detect small changes, then commonalities between the two contrasts might have been missed. Field or quantitative resistance is usually likened to basal immunity and some of the transcriptional factors identified here have been found in other pathosystems to be associated with basal resistance (Knoth et al., 2007). In particular, the NAC family of transcription factors has been found in other pathosystems to be associated with stress responses, including host responses to infection (Xia et al.; Lin et al., 2007; Bu et al., 2008). Interestingly, the putative soybean NAC domain protein transcripts (Gma.1649.1.A1_at and GmaAffx.57970.2.S1_at, representing homeologous genes) were significantly and highly expressed in the resistant cultivar Conrad relative to the susceptible line VPRIL-9, an indication that they may be associated with

regulation of defense in the resistant cultivar. Mapping these identified putative regulatory genes to linkage groups and relating them to loci associated with quantitative resistance is a further step to assessing their potential contribution to the resistance. Several transcripts including the NAC proteins mapped to important linkage groups containing loci associated with defense. One (GmaAffx.5966.1.A1_at; similar to Hypothetical protein SHOOT2) fell within a QTL mapped in V71-370 x PI407162 (MLG J, 65-95 cM), while two (GmaAffx.85063.1.S1_at, Class III HD-Zip protein 1; and GmaAffx.40296.1.A1_at, Zinc finger protein CONSTANS-LIKE 15) fell close to a QTL mapped in Conrad x Sloan (MLG L, 33-37 cM). The other genes are not ruled out from contributing to resistance. The differences in their regulation may be controlled by QTL that were not detected in the two crosses, or they may be controlled by interactions among the mapped QTL. Future research on these putative resistance regulatory genes, especially the soybean NAC domain proteins, should provide important clues about their specific roles in resistance to *P. sojae*. The mapping of these genes may prove useful for breeders searching for novel sources of resistance.

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Chapter 6

G.A.M.E.S^2 Camp: Transdisciplinary Team Education and Outreach in action

Introduction

Topic Area: Transdisciplinary education, Team Science Education and Outreach

In funding, research, practice and in scholarly writing the transdisciplinary and interdisciplinary terminology have been used interchangeably (Hall et al., 2008). To clarify the distinction between the two, the scientific orientation of transdisciplinarity is defined as:

> "a process, in which team members representing different fields work together over extended periods of time to develop shared conceptual and methodologic frameworks that not only integrate but also transcend their respective disciplinary perspectives" (Stokols et al., 2008).

Although the greater complexity of transdisciplinary collaborations make them more difficult to achieve and sustain, these collaborations have the greatest potential to produce highly novel and generative scientific outcomes (Stokols et al., 2008).

Transdisciplinary education, collaboration across disciplines in education, is an important strategy for developing new avenues of scholarly inquiry and for generating knowledge to help understand real-world problems (Wall and Shankar, 2008) that is being promoted in professional and academic circles (Wall and Shankar, 2008).

The K-12 education reform plan known as the "Paideia Proposal" was proposed by Mortimer Adler and declares that all children deserve to receive a basic quality education (Adler and Paideia, 1982). We examine an outreach program which integrates transdisciplinary education and embodies the teaching and learning styles of the Paideia Proposal.

Review:

The Paideia Proposal involves three components that promote the same course of study for all students. There are different modes of learning by students and different teaching styles (Adler and Paideia, 1982). Adler describes three different modes in which the mind can be improved by specific means. The first mode is acquisition of organized knowledge and comprises the "didactic" or "teaching by telling" mode of instruction or lecturing (Adler and Paideia, 1982). The second mode is comprised of the development of skill and here the student learns *how to do*, this mode is currently the backbone of basic schooling or coaching, where students acquire skills (Adler and Paideia, 1982). The third mode is called the Socratic method, the goal of which is for students to receive an enlarged understanding of ideas and values. The Socratic mode of teaching can also be called "maieutic" because during this mode students birth ideas through the fostering of discussion and the raising of questions (Adler and Paideia, 1982).

There is a need for transdisciplinary education. To encompass all types of knowledge about an issue, subject or idea, thinking from a transdisciplinary point of view forces one to think beyond, across and through the academic disciplines (Ertas et al., 2003). Dracup and Byran-Brown believe most professional education is discipline-specific and that few professionals have received training in collaborative and team skills (Otis-Green, 2007). Furthermore, many difficulties face educational programs because technology is rapidly changing in the environment today (Ertas et al., 2003).

> "Transdisciplinary collaboration leads to the development of shared concept frameworks that not only integrate but also transcend the individual disciplinary perspectives represented by various members of the research team" (Mâsse et al., 2008)

To better understand and enhance the processes and outcomes of scientific collaboration, the emerging field of the science of "team science" draws together diverse disciplines (Hall et al., 2008). Analyzing research questions about a particular phenomenon by promoting approaches which are collaborative and often cross-disciplinary, are the goals of team science (Stokols et al., 2008). The science of team science field encompasses an extensive array of strategies and research projects aimed at better evaluating the influences of large scale team science initiatives (Stokols et al., 2008).

Participatory action research (PAR) focuses on the needs of society and actively involves local residents on an equal basis with trained university scholars through the duration of the process (Reardon, 1998). Social learning processes are promoted throughout investigations by PAR researchers to develop communication, organizational and analytical skills of community-based organizations and their local leaders (Reardon, 1998). Transdisciplinary action research is viewed as a topic of scientific study in its own right to achieve a more complete understanding of prior collaborations and to identify strategies for refining and sustaining future collaborations among researchers, community members and organizations (Stokols, 2006).

New contribution:

Transdisciplinary Team Science is a process in which team members representing different fields work together. Getting Ahead in Math, English, Science, and Social Studies (G.A.M.E.S^2) Camp represents a transdisciplinary approach on many levels because of the diversity and collaboration among researchers from diverse disciplines, volunteers, and members of the community.

G.A.M.E.S^2 Camp began from a vision night at St. Paul African Methodist Episcopal (AME) Church, located in the Blacksburg, Virginia community. Members of the church were interested in reaching out to the children in the Greater Montgomery County community, who had little to no opportunities for activities in the summer.

St. Paul African American Episcopal Church is highly visible in the community and serves the Blacksburg Community, Virginia Tech students, staff and faculty. The outreach ministry is extensive and includes helping the homeless and needy, providing shelter, paying college rent and tuition and much more.

As a member of the St. Paul AME Church family, I have been involved with numerous leadership roles ranging from youth Sunday leader, participating on the missionary board, to leader of the St. Paul AME Women's Day Celebration. As a member of the Virginia Tech (VT) university community I have also held many leadership roles in the graduate community such as President of the Black Graduate Student Organization and Vice President of the Graduate Student Assembly. I have had the opportunity to sit on numerous commissions and committees

and was also selected to represent the VT graduate student community as a member of the race-in-the-institution task force. As a graduate student leader, I helped provide a bridge between the church community and the VT university community.

The Graduate Student Assembly (GSA), the Black Graduate Student Organization (BGSO), and St. Paul AME Church created G.A.M.E.S^2 Camp. G.A.M.E.S^2 served as a four week program with grade-specific curricula designed to accommodate 15-20 youth per grade (9^{th}-12^{th} grade). Youth from ages 13-18 were able to participate in the program and were challenged through academic and team building activities such as individualized tutorial sessions, field trips to high tech areas of the Virginia Tech campus, guest presenters, tips on behavior and etiquette, and mentoring partnerships. Students were even guided in producing their own newsletter to share the experience with others. The camp recruited over 60 volunteers from the student body of Virginia Tech, the greater Montgomery County community, and other churches. Table 6.1 lists the diverse contributors to the G.A.M.E.S^2 Camp, and their contributions.

Table: 6.1 The G.A.M.E.S ^2 Camp contributors.

Community Support	Contributions
St. Paul African Methodist Episcopal Church	G.A.M.E.S.^2 Camp innovator, provided the location and meals,
Graduate Student Assembly, Virginia Tech	Members developed curriculum, volunteer recruitment
Black Graduate Student Assembly, Virginia Tech	Members developed the education and outreach component, volunteer recruitment
National Association for the Advancement of Colored People (NAACP)	Donation
Christ Episcopal Church	Provided the building for learning
Student Leadership Employment (SLEP)	Members developed the curriculum, volunteer recruitment
Kids on Track	Donation
Educators, university officials, and leaders	Support, volunteers
Virginia Tech undergraduate students	Volunteers (mentors, lectures, activities)
Virginia Tech graduate students	Volunteers (mentors, lectures, activities)
Multicultural Academic Opportunities summer program (MAOP)	Volunteers (mentors, lectures, activties)
Ronald E. McNair Post-baccalaureate Achievement Program (McNair Scholars Program)	Volunteers (mentors, lectures, activities)
Surrounding church leaders	Volunteers (mentors, lectures, activities)

Surrounding church members	Volunteers (mentors, lectures, activities, cooking, developing educational packages)
Pan-Hellenic Council (NPHC) sorority and fraternity members	Volunteers (mentors, lectures, activities)
Interfraternity (IFC) council members	Volunteer (mentor, lecture, activities)
News Weekend Anchor, Channel 7	Presenter, taught lesson on communication
Department volunteers	
Virginia Bioinformatics Institute University Visualization and Animation Group of the Institute for Critical Technology and Applied Science (VT-CAVE) Virginia College of Osteopathic Medicine Virginia-Maryland Regional College of Veterinary Medicine Geology Department	All department volunteers arranged locations and worked with faculty, staff and graduate students to ensure students would have access to state of the art technology and receive a memorable educational experience.
Community Sponsors	
Wades Super Market	donations
Kroger	donations
Target	donations
University Bookstore	donations to complete the educational packets
Walmart	donations

Members of multiple institutions volunteered their time to participate in the G.A.M.E.S^2 Camp experience. These included VT-CAVE, the Virginia College of Osteopathic Medicine, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Bioinformatics and the Geology Department. According to the Paideia Proposal there are three different modes in which the mind can be improved. The second mode of the Paideia Proposal focuses on the development of skill. G.A.M.E.S^2 Camp provided students with the opportunity to actively gain exposure to state of the art technology and to exemplary professors in their field of study. They completed hands-on projects and were encouraged to reflect on what they have learned immediately following each exercise. This provided the students an opportunity to receive non-traditional instructional exposure, in which the students left the traditional setting of a high scholl classroom and received instruction on a university campus.

Recruitment of G.A.M.E.S^2 Camp participants was focused on neighborhoods where youth had little to no opportunities for educational activities in the summer. Volunteers knocked on doors

in trailer parks and apartment neighborhoods. Initially people did not appreciate the immediate motivation of the outreach program to involve their children. For example, parents were unaware of this type of program and couldn't believe we were offering free services to them. As conversation progressed people became a little less apprehensive and wanted to know in detail what the program entailed. The G.A.M.E.S^2 camp executive board felt it was important to connect with the individuals because of the non-traditional, one of a kind, program being offered to the community. In addition, to reach the target audience, flyers were posted in community laundry facilities, vendor bulletin boards, local schools, listserves, restaurants, churches, and community recreational facilities.

To solicit community volunteers and sponsors, letters were distributed to educators, university officials and leaders throughout the Virginia Tech campus. Letters were also distributed to community vendors, the local school board and neighborhood churches.

When: The G.A.M.E.S^2 outreach program was a four week program from July 9 to August 6, 2005.

What: G.A.M.E.S^2 camp was principally intended to enhance performance on Standards of Learning (SOL) tests. More than that however, it acted as a holistic program that exposed youth to broader opportunities and promoted the value of cultural diversity. Since youth who need this type of program often cannot afford to attend, G.A.M.E.S^2 did not charge any fees to youth and their families.

Orientation. July 9, 2005. The Kick Off Celebration "Blockin' for Jesus" served the orientation for the community in which the program overview and registration took place.

SOL Lessons. The standards of learning activities were taught once a week, on Mondays evenings. Each evening included a 30 minute break for a nutritious dinner and fellowship provided by G.A.M.E.S^2 Camp free of charge. Many volunteers were excited about the opportunity to participate in the program and were happy to dedicate one day a week to the program. Table 6.2 lists the educational activities aimed at the SOL requirements.

Table 6.2 Weekly G.A.M.E.S^2 lessons

Date	Section 1	Section 2
July 11, 2005	Math Order of Operations (P.E.M.D.A.S)	English Parts of speech, tenses, word, word order, syntax
July 18, 2005	Science experimental development (variable, hypotheses)	History Key persons in math, science, arts, government
July 25, 2005	Math conversions and graphs	English reading comprehension, writing, vocabulary, abstraction
August 1, 2005	Science organisms and data analysis (pathogens and hosts)	History Key events in math, science, art and government

Newsletter Project. G.A.M.E.S^2 met once a week, on Thursday evenings, for the newsletter project, teambuilding and community awareness. Each activity took a maximum of two hours. Table 6.3 shows the diversity among the activities. Dinner was also provided.

Table 6.3 Newsletter project, teambuilding and community awareness activities

Date	Activity
July 14, 2005	Project introduction by production groups 30 minutes each Creative writing Photography News/writing/reporting format/ advertising
July 21, 2005	Presentation on interviewing for information Students break into production groups to begin newsletter project assignment
July 28, 2005	Presentation on career skills Students break into production groups to work on newsletter project
August 4, 2005	Compile material for final cut Produce and discuss distribution plans

Weekend Outings

G.A.M.E.S^2 Camp met each Saturday for outings Table 6.4 lists the weekend activities.

Table 6.4 Weekend outings

Date	Activity
July 16, 2005	Cave Dwellers- The Cave Lab Virginia Tech Virtual Reality Lab
July 23, 2005	Dr. Bones The VCOM anatomy lab cadavers presentation
July 30, 2005	Water Roots VT campus geology Lab
August 6, 2005	Scoping it out VT Bioinformatics Lab Aquaculture Center

Awards Ceremony August 2, 2005

All participants and volunteers of the camp were invited to attend an awards ceremony hosted by St. Paul AME Church and members of the Graduate Student Organization and the Black Graduate Student Organization. The ceremony highlighted each student's commitment and dedication to the program. It was also an opportunity for the participants and the volunteers from various institutions to share fellowship one final time.

Example of email distributed to all volunteers,

> "Hello All, :-)
> The G.A.M.E.S2 Camp Team would like to thank each of you for your participation in making the Kick Off year a success. As we approach the end of the camp, we would personally like to invite you all to the volunteer appreciation dinner on Tuesday August 2, 2005 beginning at 7:00pm. There will be food, games, music, presentation of appreciation certificates and lots of FUN!! Please respond regarding your attendance. We look forward to hearing from you all soon. Sincerely,
>
> LaChelle Waller and the G.A.M.E.S2 Camp Team"

Results: Based on observation, this was the first time an educational outreach program of this magnitude was completed in Blacksburg involving the community churches, Virginia Tech University and the surrounding community. The program crossed many social and economic boundaries and allowed graduate students and undergraduate students to work in a setting in which everyone had equal identities, meaning race was not considered when interacting with G.A.M.E.S^2 Camp participants. All volunteers were welcome. Due to this environment, many ideas were discussed aimed at making the camp run more efficiently. The object of transdisciplinarity is to encompass all types of knowledge about an issue, subject, or idea while thinking beyond, across and through the academic disciplines (Ertas et al., 2003). From my perspective as the education and outreach coordinator, it was important for volunteers to have diverse educational backgrounds, and diverse pedagogy, for this would aid to developing a transdisciplinary education environment and display the connection between the diversity of the facilitators within a transdisciplinary environment. The students were able to interact with volunteers across multiple disciplines and the volunteers were able to interact in a transdisciplinary environment through working collaborations within the community. Weekly lessons were carried out at the churches while weekend activities were conducted within various departments on the Virginia Tech campus. Many volunteers were from several different

disciplines within the university such as, engineering, physics, social sciences, interdisciplinary, biology, education, and psychology.

Discussion

Transdisciplinarity is the process through which researchers collaborate to develop a shared theoretical structure that extends and integrates theories, methods and discipline-based concepts used to address a common topic of research interest (Stokols, 2006). Transdisciplinary research collaborations are intended to achieve the highest levels of intellectual integration across multiple fields to yield shared conceptual formulations, that move well beyond individual team members' disciplinary perspectives (Stokols, 2006).

Based on observations, the G.A.M.E.S^2 Camp outreach program displayed the true meaning of transdisciplinarity. The camp included diverse researchers from many educational backgrounds, volunteers from within the community throughout the New River Valley and from various university summer outreach programs at Virginia Tech, crossing many cultural boundaries. G.A.M.E.S^2 Camp provided more of an enquiry-based approach to learning, as opposed to students memorizing instructional materials. The volunteers acted as facilitators of the students' learning process. As such the G.A.M.E.S^2 Camp meets the primary goal of Transdisciplinary Team Science because it is was a process in which team members representing different fields worked together on a common activity that was a synthesis of each contribution.

As mentioned above, participatory action research (PAR) focuses on the needs of society and actively involves local residents on an equal basis with trained university scholars through the duration of the process (Reardon, 1998). G.A.M.E.S^2 Camp actively involved diverse members of the Blacksburg community, especially the church community, as well as university scholars. Furthermore, G.A.M.E.S^2 camp was focused on the educational needs of under-served youth in the community. As an ambitious experiment in how to reach out to youth who would normally not have access to educational enrichment programs, G.A.M.E.S^2 Camp also constituted a research project. In these ways therefore, G.A.M.E.S^2 Camp represents an example of PAR.

Outreach programs can receive much added value by practicing transdisciplinary team science, in which a diversity of researchers volunteer to participate in a program. A common successful outreach model has been the "scientist in the classroom" (Laursen et al., 2007). The "scientist in the classroom" model is oriented specifically to science education, and in the case of G.A.M.E.S^2 Camp contributed tremendously to the science component of the camp. However, G.A.M.E.S^2 Camp expanded this concept beyond the STEM fields, adding analytical skills, English, Social Studies, and life skills. The camp created exciting opportunities for meeting SOL learning goals because of its relevance and ability to appeal to students through hands-on classroom activities.

Observing the camp from beginning to end, the program began with students who were not interested in participating in the camp. Most students became participants because of enthusiasm from their parents. By the end of the program the attitude of the students completely changed. This was particularly noticeable through the enthusiasm of the students in creating the student newsletter. The newsletter was a huge success. The students were able to reflect upon their experience at the G.A.M.E.S^2 camp. All students were required to work together to complete the components of the document. The students made up the editorial board, as well as the writers and photographers, while receiving support on editorial advising and design.

The outreach program embodied a transdisciplinary team approach. The program valued the knowledge and skill of each team member. The transdisciplinary team approach depends on effective and frequent communication among members, and it promotes effectiveness in the delivery of educational services. Members of the transdisciplinary team share knowledge, skills, and responsibilities across traditional disciplinary boundaries in assessment and planning. Transdisciplinary teamwork involves a certain amount of boundary blurring between disciplines and implies cross training and flexibility in accomplishing tasks (DYER, 2003).

G.A.M.E.S.^2 Camp displayed the importance of change and stepping out of the box, in several ways. One of these was the fact that the program was initiated and led by a graduate student from the Virginia Bioinformatics Institute (VBI), which is a transdisciplinary research institute. G.A.M.E.S.^2 camp made a large and valuable contribution to the outreach mission of VBI. From the point of view of the community, G.A.M.E.S^2 Camp served as an innovative outreach program and an extraordinary opportunity to build and unify a community. The community

became a source of value for the campus. From the point of view of the campus, there was a vision for the campus and outreach took place across multiple departments due to the participants who volunteered their time and educational scholarship. To have a program with such diversity take place in Blacksburg Virginia and at Virginia Tech expressed change over time. This was once a community where blacks were not allowed on campus. There were many elderly members who participated in G.A.M.E.S^2 Camp who expressed their sincere appreciation for this dimension. Change evolved through the vision of the St. Paul AME church, of Virginia Tech graduate students, and of community members including church members, and parents who wanted a positive outlet for their children. This program was larger than the community because the participants involved were on a challenging path and did not have many community options to stay clear of the juvenile justice system. The level of outreach was not compartmentalized nor directed to the affluent, making it a huge success to so many.

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