CYTOSINE METHYLATION OF PHYTOPHTHORA SOJAE BY METHYLATED DNA
IMMUNOPRECIPITATION

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ABSTRACT

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Phytophthora sojae undergoes many developmental changes throughout its lifecycle from freely swimming zoospores to vegetative mycelium. These changes in development are accompanied by changes in gene expression; however, little is known about gene regulation in this organism. During infection effector proteins are secreted and act as one of the major virulence determinants of *P. sojae*. Avirulence genes have been long sought after because these genes control race-cultivar compatibility. Different strains of *P. sojae* have been shown to carry copies of the same effectors but display variation in expression; gene silencing mechanism of some of these genes is not known.

One common epigenetic mechanism used to regulate gene expression by both prokaryotes and eukaryotes is DNA methylation, however; the DNA methylation status of the soybean pathogen *P. sojae* has yet to be shown. We provide evidence of cytosine methylation in the promoter regions of two known *Avr* effectors, Myb DNA-binding proteins, and the housekeeping gene actin by the method of MeDIP. DNA methylation was detected in a range of genes suggesting that *P. sojae* employs DNA methylation as one mechanism of gene regulation.

Dedicated to my loving and supportive family.

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INTRODUCTION

Plant Pathogenic Oomycetes

Oomycetes are a diverse group of organisms that are saprophytes or pathogens of a variety of host including plants, algae, insects, fish, crustaceans, mammals, and microorganisms (Kamoun, 2003). Phylogenetically, oomycetes are members of the kingdom Chromista or belong to the Stramenopiles (Van de Peer *et al*, 1997). Oomycetes are often referred to as fungi-like because of their filamentous growth habit, modes of nutrition, and ecological roles; however, they are evolutionarily distant from true fungi (Tyler, 2007; Richards *et al*, 2006). Oomycete cell walls are mainly composed of β-1,3-glucan polymers and cellulose, unlike fungal cell walls, which are mainly composed of chitin (Erwin *et al*, 1996). Nonetheless, chitin synthase genes are widely distributed among oomycetes and the chitin synthase inhibitor nikkomycin Z caused reduction in growth in *Saprolegnia* (Guerriero *et al*, 2010). This indicates chitin is a minor but important part of the oomycete cell wall. Other closely related members of the Stramenopiles include gold-brown algae, diatoms, and brown algae such as kelp (Tyler, 2009).

The Genus *Phytophthora*

The genus *Phytophthora* contains more than 80 species of oomycete plant pathogens, which occur in both natural and agricultural settings (Erwin *et al*, 1996). Members of this genus are most well known for their enormous economic damage to important crops such as potatoes, tomatoes, soybeans, and alfalfa (Kamoun, 2003). Unique biological features

common to *Phytophthora* species are coenocytic mycelium with few or no septa,

zoosporangia that release biflagellate zoospores in water, and sexual reproduction

through the production of oospores (Erwin *et al*, 1996). *Phytophthora* species do not

synthesize sterols and require an exogenous source of β-hydroxyl sterols for sporulation

(Erwin *et al*, 1996). Some species such as *P. cinnamomi*, *P. parasitica,* and *P. ramorum* have

very broad host ranges, while others such as *P. sojae* and *P. infestans* have narrow host

ranges (Erwin *et al*, 1996). Infection strategy is also highly diverse in this genus, ranging

from species specialized in colonizing leaf litter to highly specialized pathogenic

hemibiotrophs (Hein *et al*, 2009).

Phytophthora sojae

    Phytophthora sojae is a hemibiotroph oomycete that primarily infects soybean,

causing root and stem rot. *P. sojae* can also infect many lupine species native to North

America (Jones, *et al*, 1969). The economical damage caused by *P. sojae* is approximately

$200 million in annual losses to the northern Midwest of the USA, and worldwide damage

is estimated around $1-2 billion (Tyler, 2007). This destructive pathogen remains to be the

most damaging problem that confronts soybean producers. *P. sojae* has a genome size of 95

Mbp and is diploid throughout most of its life cycle.

    Management of *Phytophthora* root and stem rot is predominately handled through

the deployment of race-specific cultivars. Pathogens and host plants interact in a gene-for-

gene system. Specific resistance genes (*Rps*) of the soybean host will show resistance to *P.*

sojae strains only if they carry the corresponding avirulence (*Avr*) genes (Dorrance *et al*,

2009). For example, a soybean cultivar containing the resistance gene *Rps*1a is only protected against *P. sojae* strains that possess the cognate *Avr*1a gene (Qutob *et al*, 2009). Soybean *Rps* genes are believed to activate effector-triggered immune responses and provide absolute protection against infection. There are at least 14 known *Rps* genes that localize to four different multiple linkage groups in soybean (Demirbas *et al*, 2001). Other methods of control, include chemical treatments of seeds with fungicides such as metalaxyl; however, this approach is costly and only provide minimal control of disease (Gijzen *et al*, 2009).

In the United States, it is believed that *P. sojae* populations have adapted to several of these *Rps* genes and that these genes are no longer effective at providing resistance in some regions (Boerma *et al*, 2004). An earlier study conducted by Schmitthenner *et al.* (1994), documented changes in virulence composition of *P. sojae* populations in Ohio from 1980 to 1990. From 1978 to 1980, they reported the majority of isolates were classified as *P. sojae* race 7 (virulence for *Rps* 1a, 3a, 7), race 9 (1a, 6, 7), and race 3 (1a, 7). In 1990, the majority of isolates were classified as races 3, 7, 4 (1a, 1c, 7), and 1 (7). In the following year, 1991, races 3, 7, and 9 were the most common. In total 18 new races of *P. sojae* were identified. This increasing number of races and adaptation to deployed *Rps* genes was also observed in Illinois, Indiana, Missouri, North Dakota, and South Dakota. The authors concluded that *P. sojae* was a highly variable pathogen with regards to virulence phenotype. Pathotype diversity among *P. sojae* may develop alone or in combination with mutation, outcrossing, and mitotic recombination (Goodwin, 1997).

Infection Process and Life Cycle

Disease caused by *P. sojae* usually begins below the soil line and spreads up the stem; however, aerial parts of the plant can be directly infected by rain splashes (Tyler *et al*, 2007). In mature plants, root and stem rot is observed in the field as well as damping off of germinating seedlings (Tyler, 2007). The life stages of *P. sojae* are shown in Figure 1. Asexually produced zoospores are the main route of infection and are dispersed in the soil when conditions are wet. Up to 30 zoospores are released from a single sporangium (Tyler, 2002). These biflagellate water-motile spores are attracted to isoflavones and other compound secreted by soybean roots and will swim chemotactically toward their host (Morris and Ward, 1992). Upon locating the host, zoospores will rapidly secrete materials to make a temporary wall and shed their flagella. This cyst is quite adhesive and attaches firmly to the root surface (Tyler, 2002). Invasion of host tissue begins with cyst germination and the resulting germ tube can either directly penetrate the host or grow along the plant surface. Appressorium swelling occurs to apply pressure on the plant surface and assist in physical host penetration (Hardham et al, 2009). *P. sojae* hyphae will initially remain in the apoplast of host cells where specialized feeding cells called haustoria will develop to obtain nutrients from host tissue during the biotrophic phase of infection (Hardham and Shan, 2009). During a compatible interaction, direct cell penetration occurs later during infection as the pathogen switches from a biotrophic to a necrotrophic lifestlye. The necrotrophic stage is characterized by massive proliferation of invading hyphae within the stele. Due to cell invasion, the infected plant tissue will lose

turgor and develop water-soaked lesions. Throughout the infection process, gene

expression patterns in the host and pathogen change rapidly (Hardham *et al*, 2009).

Sexual reproduction of *P. sojae* occurs in the female organ called the oogonium and

the male organ called the antheridium. The antheridium will fuse with the oogonium and

transfer a single haploid nucleus (Tyler, 2007). The fertilized oogonium will differentiate

into an oospore, which can survive in the soil for long periods of time. These large sexual

oospores are abundantly produced in infected tissue (Judelson *et al,* 2009). There are no

known mating types of *P. sojae* and it is mainly considered to be homothallic; however,

outcrosses can occur between different strains, which provides sources of variation
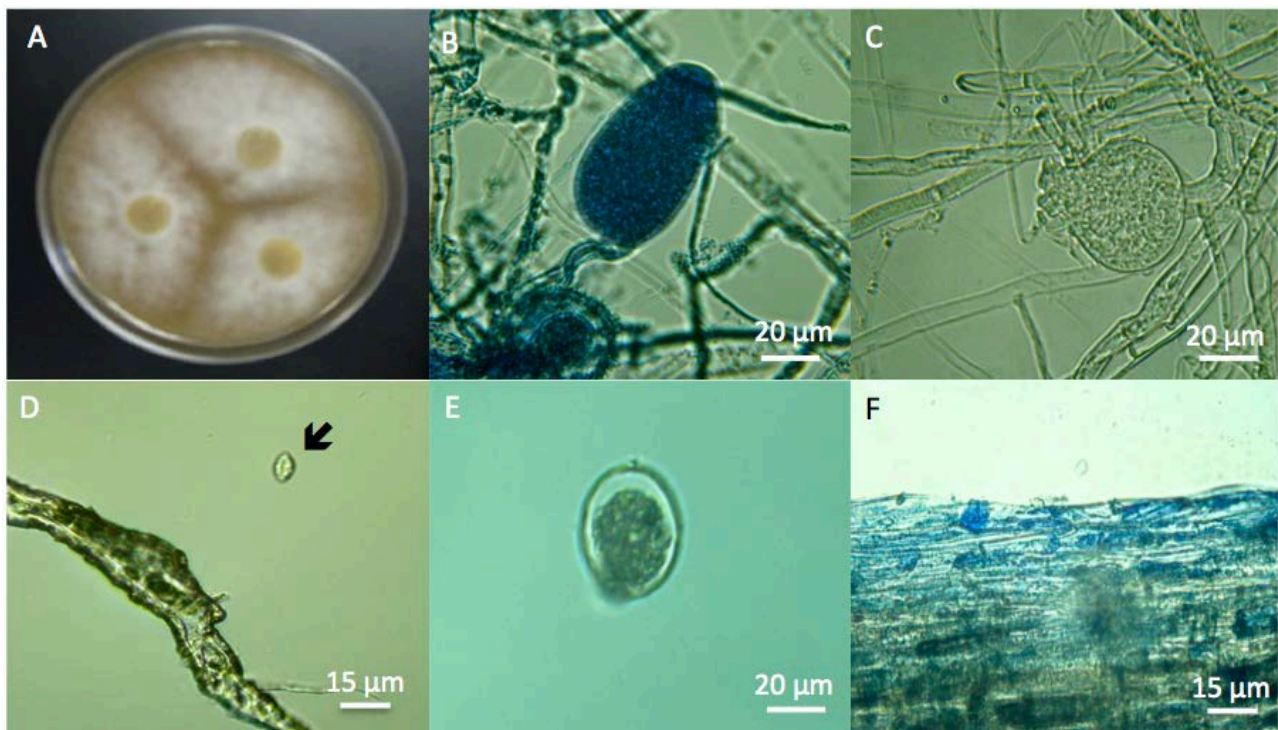
(Förster et al, 1994).

Figure 1. Life stages of *Phytophthora sojae*: A) *P.* sojae mycelium grown on V8-agar. B) Asexual sporangia stained with lactophenol cotton blue formed at the apex of somatic hyphae. C) A bursting sporangia; up to 30 zoospores will be released. D) An ovoid shaped motile zoospore, indicated by the arrow. Biflagellate zoospores are released in the soil when conditions are wet. E) An oospore showing thick cell wall; these asexual resting spores can survive in the soil between soybean growth seasons. F) Williams 82 (resistance) soybean hypocotyl at 6 hours post infection with *P. sojae* hypha. Mycelium is stained with lactophenol cotton blue (100× magnification).

The success of oomycetes as pathogens depends on their ability to overcome the defenses of their host. The plant hosts have complex multifaceted defense systems that include cell wall reinforcement, programmed cell death, phytoalexins synthesis, and reactive oxygen species (ROS) generation (Lamour *et al*, 2006; Jones *et al*, 2006). In order to complete their lifecycle, *P. sojae* must be able to establish an intimate association with the host plant cell and obtain nutrients from the host during the biotrophic phase. Oomycetes are able to establish this relationship by reprogramming the defense response of host cells through an array of effector proteins (Kamoun, 2006). Effectors proteins can manipulate host cell structure and function thereby establishing infection and/or triggering defense responses (Kamoun, 2006).

PAMP Triggered Immunity

The front-line of defense encountered by a pathogen is the plant non-self surveillance system that perceives attempted invasions (Ingle et al, 2006). Pattern recognition receptors (PRRs) located on the plant cell surface detect conserved molecules such as PAMPs (pathogen associated molecular patterns) that are secreted or displayed on

the surface of microorganisms (Hein *et al*, 2009). PAMP-triggered immunity (PTI) constitutes the plant innate immunity and induces a broad-range of defenses (Hein *et al*, 2009). One of the first proteinaceous oomycete PAMPs to be indentified is a calcium-dependent transglutaminase that functions in the cell wall of *P. sojae* (Brunner et al, 2002). Other *Phytophthora* PAMPs known to induce PTI are elicitins, glucans, cellulose-binding proteins, and a number of small cystein-rich proteins (Kamoun *et al,* 2006).

Effector Triggered Immunity

Plants have a second line of defense that acts largely inside the cell in the form of resistance proteins. Many resistance genes in plants encode intracellular receptors that are capable of detecting the presence of effectors produced by the pathogen, termed avirulence proteins (Avr) (Lamour *et al*, 2006). Many of these receptors consist of proteins with nucleotide binding sites (NBS) and leucine-rich repeats (LRR) (Lamour *et al*, 2006). These NBS-LRR proteins have intracellular locations and upon detection of avirulence proteins, a defense response termed effector-triggered immunity (ETI) is initiated. ETI is an accelerated and amplified PTI response that results in a hypersensitive cell death at the site of infection, thus providing disease resistance (Jones *et al*, 2006). The intracellular location of resistance genes implies that oomycetes must also introduce effectors into the cytoplasm of their host (Lamour *et al*, 2006). This lead to the discovery of a large superfamily of effector proteins in oomycetes. Members belonging to this class of effectors share the protein motif RXLR-dEER (arginine, any residue, leucine, arginine- aspartate (low frequency), glutamate, glutamate, arginine) (Lamour *et al*, 2006). This motif is located

downstream from the signal peptide in the N-terminal of the protein and allows the protein to translocate into the cytoplasm of the host cell.

The C-terminal of RXLR effector proteins contain the domain with biochemical effector activity and displays higher levels of polymorphisms and signatures of positive selection (Schornack *et al*, 2009). Because effectors are major virulence determinants that can be recognized by plant R proteins, effector repertoires are thought to be shaped through a coevolutionary arm race (Kamoun, 2007). An evolving pathogen will place increasing pressure on its host as its virulence increases. This will result in an improvement in the host's resistance mechanisms. In turn, this places selective pressure on the pathogen to adapt or lose the host (Tyler, 2009).

There are more than 400 predicted RXLR effector proteins in the *P. sojae* genome. These effectors have been proposed to interact physically in a gene-for-gene system with resistance (R) genes of the host (Shan *et al*, 2004). Effectors with avirulence activity are recognized by host cell *R* proteins, resulting in the induction of hypersensitive cell death and immunity (Schornack *et al*, 2010). Other virulent effectors can evade host detection and function to suppress host cell immunity. This allows the pathogen to keep the host cell alive during the biotrophic stage of infection, allowing the pathogen to gain nutrients. During the infection of host plants, effector genes display patterns of tight temporal regulation (Schornack, *et al*, 2010). For example, several effectors of *P. infestans* are transcriptionally upregulated during the pre-infection and early stages in host infection (Whissson *et al*, 2007). During the biotrophic stage, at least 79 RXLR effectors with known

avirulence activity were sharply induced until 2-3 days post-inoculation (Haas *et al,* 2009). Later during the necrotrophic phase, toxins such as NPP1 are expressed (Kanneganti *et al,* 2006). Tight temporal regulation of effector genes results in distinct stage-specific expression patterns. This reflects the intricate processes of cellular control exhorted by *P. infestans* as effectors are deployed during host colonization (Schornack *et al,* 2010).

Avr1a of *P. sojae*

Two well-characterized avirulence effectors of *P. sojae* are *Avr1a* and *Avr1b*. Map-based cloning of the *Avr1a* locus by Qutob *et al.* (2009) revealed two identical copies named *Avh275a* and *Avh275b* (avirulence homolog) and two additional homologs named *Avh275c* and *Avh72*. The *Avh275c* homolog contains polymorphisms in the 3' region and further analysis of *Avh72* gene revealed that it encodes a pseudogene because the open reading frame is interrupted by a premature stop codon (Qutob *et al,* 2009). Copy number variation of *Avh275* (*Avh275a* and *Avh275b*) and *Avh275c* differs among different *P. sojae* races. Southern blot analysis showed race 2 contains a gene cluster consisting of the *Avh72* pseudogene, *Avh275c,* and two identical copies of *Avh275*. Many other isolated races, such as race 7 contained *Avh72* and *Avh275c* but lacked copies of *Avh275* (Qutob *et al,* 2009). Most surprisingly, Southern blot analysis with an *Avh275* probe revealed that *P. sojae* races 12, 19, and 25 contain copies of *Avh275*; however, transcripts do not accumulate in host cells. The mechanism of gene silencing at the *Avr1a* locus among different strains of *P. sojae* is not known (Qutob *et al,* 2009).

Avr1b of *P. sojae*

Map-based cloning and sequencing of the *Avr1b* locus in *P. sojae* identified two genes, *Avr1b-1* and *Avr1b-2*. Shan *et al* (2004) demonstrated that one of the genes (*Avr1b-1*) encodes for an avirulence factor and the other gene (*Avr1b-2*) controls the accumulation of *Avr1b-1* mRNA. In some strains of *P. sojae* that are virulent to *Rps1b* cultivars, such as P6497 and P9073, the sequence of *Avr1b* gene was identical to that of an avirulent strain, *P6954*; however, no mRNA from the *Avr1b* gene could be detected. Further sequencing showed that there were no differences in P6497 within a 4.9-kb region upstream to the gene, nor within 0.4-kb downstream (Shan *et al*, 2003). In other virulent strains such as P7081, P7076, and P7074, a large number of amino acid substitutions were present in the coding region of *Avr1b*, suggesting that loss of the Avr1b$^+$ phenotype was due to these mutations (Shan *et al*, 2003).

Further analysis of *Avr1b* in P6497 revealed that the lack of *Avr1b-1* mRNA was due to the lack of a trans-acting factor, *Avr1b-2*. The trans-acting factor was discovered in a cross between P6497 and the transcriptionally active P7064 allele. mRNA was collected from 4 F$_2$ progeny and digested with *Age1*, an enzyme that detects the single polymorphisms that distinguishes the two alleles (Shan *et al*, 2004). In all 4 heterozygous progeny, the mRNA of P6497 allele was represented equally as the P7064 allele from *P. sojae* infected soybean tissue. This demonstrates that the lack of *Avr1b*-1 mRNA in P6497 is due to the lack of a trans-acting factor (*Avr1b-2)* which can be provided by the P7064 genome (Shan *et al*, 2004). *Avr1b-2* was genetically mapped to the same BAC contig as *Avr1b-1* and functions to elevate *Avr1b*-1 mRNA by either stimulating *Avr1b-1*

transcription or acting to stabilize *Avr1b-1* mRNA (Shan *et al,* 2004). Regardless, loss of effector gene transcription may be a common mechanism of evading R-gene mediated immunity (Qutob *et al*, 2009).

Phytophthora sojae Genetics

The 95 Mb genome of *P. sojae* was sequenced by shotgun sequencing together with bacterial artificial chromosome (BAC) to an average depth of 9× (Tyler, 2006). The haploid chromosome count is not exactly known due to the presence of several small chromosomes that are difficult to resolve under light microscopy; however, there appear to be 12 to 15 chromosomes of *P. sojae*. Databases of expressed sequence tags (ESTs) have also been generated; currently there are more than 28,000 ESTs. On average transcribed sequences contain 58% GC content. EST data is extremely useful in showing gene expression patterns during different stages of the life cycle. In *P. infestans*, 15,650 genes are expressed during sporulation, of which 324 are highly expressed only in sporangia, 319 are highly expressed in sporangia and germinated cyst, and 629 are highly expressed in sporangia and cleaving sporangia (Judelson *et al,* 2008). An extensive change in gene expression has also been observed during zoosporogenesis, encystment, and the sexual life cycle.

Phytophthora genomes contain an abundance of repetitive sequences (Kamoun, 2003). In *P. sojae* five families of tandemly repeated sequences were identified and varied in copy number between isolates and were all localized on a single chromosome (Kamoun,

2003; Mao *et al*, 1996). Sequences similar to class II transposable elements are also abundant in *Phytophthora* genomes (Kamoun, 2003).

High gene density is observed in the *P. sojae* genome. Several functional genes are known to be located in high-density gene islands separated by cluster of repetitive sequences (Kamoun, 2003). Analysis of gene structure shows a majority of genes do not contain introns. The genes that do contain introns have conserved sequences at the 5′ and 3′ exon-intron junctions (5′-GTRNGT…YAG-3′) (Kamoun, 2003). Conservation around the ATG translational start codon follows the eukaryotic consensus ACC<u>ATG</u>A (Kamoun, 2003).

Promoters from non-oomycete species do not function in *Phytophthora*, suggesting the presence of unique translational machinery (Judelson *et al*, 1992). Eukaryotic promoter elements such as the TATA box were not always detected in oomycete promoters. The transcriptional start sites of several genes have been determined and are typically only 50 to 100 bp upstream of the start codon. This suggests that oomycetes have highly compact transcripts, with some of the shortest untranslated regions noted for eukaryotes (Pesole *et al*, 2000).

<u>Mechanism of Gene Silencing in *Phytophthora*</u>

Gene silencing through the RNA interference (RNAi) pathway is a common mechanism found in many eukaryotes. In many organisms where this type of posttranscriptional gene silencing operates, it can also be caused by epigenetic changes at

the DNA level, such as *de novo* methylation of cytosines or through modification of histone proteins (Lamour, 2006). Genes encoding proteins that exhibit similarity to RNAi components have been identified in oomycete databases (either EST or genome) (Lamour, 2006). Gene silencing has been extensively studied in *P. infestans*. Exogenous application of synthesized double stranded RNA (dsRNA) *in vitro* has been shown to initiate short-term silencing in protoplast of *P. infestans* for up to 15-days after exposure (Whisson *et al*, 2005). At this time after exposure, it is unlikely that any of the original applied dsRNA is still present, suggesting the silencing signal must either be secondary or amplified; perhaps through the action of RNA-dependent RNA polymerases or maintained at the DNA level through methylation or chromatin modifications (Lamour, 2006).

De novo methylation of DNA sequences does not seem to play a role in silencing of transgenes or their endogenous homologs in *P. infestans*. The digestion of DNA from silenced transformants with methylation sensitive enzymes and bisulfite-treated DNA did not show evidence of DNA methylation at the silenced loci, INF1 (West *et al*, 1999). However, contradictory results were obtained from studies using the DNA methyltransferase inhibitor, 5-azacytidine (van West *et al*, 2008). Despite the lack of detectable cytosine methylation at silenced loci, 5-azacytosine could reverse silencing in *P. infestans*. It is possible 5-azacytosine also inhibits other methyltransferase enzymes, such as histone methyltransferases and this was further explored by van West *et al* (2008). This group showed that *inf1*-silenced transformants with the histone deacetylase inhibitor trichostatin A also lead to a reversal of silencing. Thus, it has been speculated that

transcriptional gene silencing in *P. infestans* and possibly in other oomycetes, is based on the formation of non-DNA-methylation but histone deacetylated and methylated heterochromatin, which is triggered by siRNAs (Lamour, 2006).

DNA Methylation

All cells of a multicellular organism contain the same genetic material coded in their DNA sequence, but cells obviously display a broad morphological and functional diversity (Tost, 2009). This diversity is caused by differential expression of genes. Epigenetics can be defined as heritable changes in gene expression that are not caused by changes in the nucleotide sequence of DNA itself (Tost, 2009).

One epigenetic mechanism utilized by both prokaryotes and eukaryotes is methylation. In eukaryotic cells methylation is almost exclusively found at position 5 of the cytosine ring (Jeltsch, 2002). The context of DNA methylation mainly occurs at CpG dinucleotides but varies among eukaryotes. In mouse embryonic stem cells methylation has been shown to occur at CpNpGp sites and in plants methylation can also occur at CpA sites (Jeltsch, 2002; Tost, 2009). The occurrence of methylation also varies among eukaryotes. In higher plants having relatively large genomes, around 30% of cytosines are methylated, while only 1-2% of cytosines are methylated in the small genome of *Neurospora crassa* (Selker, 1990).

The patterns of methylation within each genome are not random. Certain regions are always free of methylation, others are always methylated, and some are methylated in

particular tissue or under certain conditions (Selker, 1990). In plants, DNA methylation

patterns are species-, tissue-, organelle-, and age-specific (Vanyushin *et al,* 2009).

Methylation patterns in the promoter region of the tobacco T85 gene was observed in

pollen DNA; however, this same region was unmethylated in leaf DNA (Oakley *et al*,

1996). Specific changes in DNA methylation accompany the entire life of a plant; starting

from seed germination up to programmed death (Vanyushin *et al,* 2009). Repetitive

regions also appear prone to methylation, particularly satellite regions and transposons

(Jeltsch, 2002; Selker, 1990).

Roles of DNA Methylation

Eukaryotic DNA methylation is involved in a variety of functions including

replication, DNA repair, gene transposition, transcription, and cell differentiation

(Vanyushin *et al*, 2009). The methylation state of a chromosome serves as an index of DNA

replication and only fully methylated chromosomes initiate replication (Zweiger *et al*,

1994). Following chromosome duplication the newly synthesized daughter strand is

unmodified and the DNA is in a hemimethylated state. A class of methyltransferases with

maintenance activity is responsible for reestablishing methylation patterns that are present

on the complementary strand. As a result of this, methylation patterns are stable over cell

divisions and are inherited from the parental strand (Jetlsch, 2002). A major maintenance

methyltransferase named *MET1* has been identified in *Arabidopsis thaliana*. This gene was

identified based on homology to the mouse maintenance methyltransferase, Dnmt1 that

was first described by Bestor et al (1988). *MET1* is expressed in seedlings, vegetative, and

floral tissue especially in the meristem zones (Ronemus *et al*, 1996). Inhibition of *MET1* by an antisense construct affected normal patterns of development, especially those associated with flowering. Other abnormal phenotypes included small round leaves, reduced apical dominance, and shorter roots with more branching (Finnegan *et al*, 1996).

The mobility of transposable elements is also affected by methylation. *Mutator*-like elements with long terminal inverted repeats in *Arabidopsis* strain Columbia have been shown to be dormant and not transposing (Singer *et al*, 2001). This dormant state is correlated with DNA methylation and therefore a lack of transcription. In mutants where a decrease in DNA methylation (*ddm1*) is observed, the transposon methylation was eliminated; resulting in high levels of transposition.

During transcription methylation plays a regulatory role. Methylation located at the 5′ end of genes tends to result in transcription inactivation, therefore decreasing gene expression. CpG island promoters contain a stretch of DNA with high CpG content. Approximately 60% of human and mouse genes contain promoters within dense CpG regions. Promoters of tissue specific genes containing CpG islands are methylated in most cell types, however housekeeping genes, which contain CpG islands, are constitutively unmethylated (Brandels *et al*, 1994). A CpG island in the promoter region of the housekeeping gene, adenine phosphoribosyltransferase (APRT), was shown to escape *de novo* methylation through the action of SP1 binding to sites in or near these regions (Brandels, *et al*, 1994). In contrast, Shet et al. (1999) showed a subset of male germ line-

specific genes, the MAGE-type genes, are silenced in human somatic tissue by DNA methylation in the CpG rich promoter.

Although methylation in regulatory regions tends to decrease transcription, the mechanisms of this repression can be indirect. Two models have been proposed to explain how methylation can inhibit transcription (Rountree *et al*, 1997). In the first, methylation interferes directly with the binding of trans-acting factors to their recognition sites located in promoter regions. The transcription factors AP-2 and NF-κB do not bind to their recognition sites when these sites include a methylated CpG (Comb *et al*, 1990; Bednarik *et al*, 1991). However, this mechanism of gene repression by methylation is not general. Other transcription factors such as CTF are insensitive to the methylation status within their recognition sites. Methylation of the CpG dinucleotide immediately adjacent to the HSV *tk* CCAAT-box had no effect on the affinity of CTF for its recognition site (Ben-Hattar *et al*, 1989). Even though CpG methylation of the CTF recognition site had little effect on binding *in vitro*, DNA methylation still repressed transcription. Therefore it could not be concluded that methylation inhibited transcription by a simple direct effect of on the binding of CTF to its recognition site (Ben-Hattar *et al*, 1989).

In the second model, methylation inhibits transcription indirectly by inducing a heterochromatin structure or by attracting repressor proteins that bind to methylated DNA in a sequence-independent manner (Buschhausen *et al*, 1987; Tate *et al*, 2004). Two methyl-CpG binding proteins, MeCP-1 and MeCP-2, have been identified and shown to bind only methylated symmetrical CpG dinucleotides. Binding of MeCP-1 to methylated

promoters was shown to repress transcription *in vitro* (Boyes *et al*, 1991). Gene repression

mediated by Me-CP1 depends on the level of methylation and the strength of the

promoter (Boyes *et al*, 1992). A low level of methylation to a weak promoter is sufficient

enough to reduce transcription; however, when the strength of the promoter increases,

repression is overcome (Boyes *et al*, 1992).

More recently methylation has also been shown to occur in the fungi *Neurospora*

crassa and has several unusual features. First, the context of 5-methylcysotsine was not

limited to symmetrical CpG sites nor was it located in any particular oligonucleotide

sequence (Selker, 1993). In contrast to the situation in mammals and plants, DNA

methylation is not essential in the fungus. Methylation appears to be controlled by only

one DNA methyltransferase, DIM-2 and mutations to this enzyme eliminate all DNA

methylation without causing growth defects (Foss *et al,* 1993). Most of the methylation

found in *Neurospora* is associated with sequences that have been altered by the process of

repeat-induced point mutation (RIP). RIP scans the genome looking for duplicated

sequences and once found, makes numerous GC to AT mutations (Singer *et al*, 1995). If a

gene is found within the duplicated region, the RIP process typically leaves the gene

nonfunctional and any remaining cytosines are methylated (Singer *et al*, 1995).

In prokaryotic systems, methylation of adenine was shown to be involved in the

pathogenicity of *Salmonella typhimurium*, *Vibrio cholera, Haemophilus influenza,* and *Yersinia*

pseudotuberculosis (Falker *et al*, 2005). In *Salmonella typhimurium* DNA adenine methylase

(Dam) regulates the expression of at least 20 genes known to be induced during infection.

Dam mutants showed serve defects in the colonization of deeper tissue sites and were completely avirulent in mice (Heithoff *et al*, 1999).

DNA Methyltransferases

Methylation patterns are established and modified by a group of enzymes known as DNA methyltransferases (MTases), which use S-adenosyl-methionine (AdoMet) as the methyl donor. Traditionally there are two different kinds of DNA MTases based on their methylation activity. One group of MTases, referred to as maintenance methyltransferases, shows a preference toward hemimethylated DNA and maintain methylation patterns following DNA replication (Herman *et al*, 2004). The first characterized maintenance methyltransferase was Dnmt1 of mice (Bestor *et al*, 1988). A plant homolog to Dnmt1 was characterized in *Arabidopsis* named MET1. Missense mutations to MET1 led a decrease in overall genome methylation at both CpG and CpNpG sites, resulting in abnormal phenotypes (Kankel *et al*, 2002).

Methylation itself can also be edited by either *de novo* methylation or demethylation, thus this epigenetic alteration is reversible (Jetlsch, 2002). The *de novo* class of DNA methyltransferases creates new methylation marks on the DNA and is able to methylate asymmetrical non-CpG sites. Dnmt3a and Dnmt3b are two such methyltransferases and are both highly expressed in embryonic tissue, whereas low expression is observed in adult somatic tissues (Okano *et al*, 1998). Double mutant mice lacking Dnmt3a and Dnmt3b exhibit no *de novo* methyltransferase activity and die in mid

gestation (Okano *et al*, 1999). This indicates the critical role for these enzymes during development.

Methylated DNA Immunoprecipitation

There are a number of techniques available to study the occurrence of methylcytosine in the genome. These techniques include: methylation-sensitive restriction enzymes, bisulfite conversion, methylated DNA immunoprecipitaion, affinity purification by methyl-binding proteins, and high throughput liquid chromatography. Each method has limitations to type of information that can be generated. Methylated DNA immunoprecipitation can be used to determine methylated regions on a genome wide-scale or for individual loci. DNA is randomly sheared by sonication and methylated regions are immunocaptured with bead-immobilized anti-5-methylcytosine antibodies. Methylated regions are detected without sequence information and antibodies are highly specific. The methylated fraction is washed from the beads and is suitable for use in PCR to determine the methylation status of individual regions.

5-aza-2′-deoxycytidine

5-aza-2′-deoxycytidine (5-aza-dC) is a nucleoside base analog of cytidine that contains a nitrogen atom in replace of the carbon at position 5 of the pyrimidine ring (Haff, 1995). This analog becomes incorporated into DNA where it binds to DNA methyltransferases in an irreversible, covalent manner (Santi *et al*, 1987). This inhibition causes almost complete demethylation of the genome and genes originally silenced by

hypermethylation are re-expressed after treatment with the drug. Originally 5-aza-dC

was used as an anticancer agent but the effectiveness of the drug has produced conflicting

results in different human cancer cells (Zhu et al, 2004). Nevertheless, this drug has been

frequently used to manipulate genomic methylation to demonstrate the effects of DNA

methylation on gene expression.

PURPOSE

P. sojae undergoes many developmental changes throughout its lifecycle from freely swimming zoospores to vegetative mycelium. These changes in development are accompanied by changes in gene expression; however, little is known about what controls the expression of genes in this organism. One of the major virulence determinants of *P. sojae* depends on the presence of RXLR effectors. Among different *P. sojae* strains these RXLR effectors display variation in expression even though the gene is present. The mechanism of silencing of certain RXLR effectors remains unknown.

We hypothesize that cytosine methylation is one mechanism utilized by *P. sojae* to regulate the expression of its transcripts. The purpose of this experiment was to examine the effects of DNA methylation on gene regulation in *P. sojae* using methylated DNA immunoprecipitation (MeDIP). qRT-PCR was employed to measure the level of transcripts of three genes containing Myb DNA-binding domains, two genes containing RXLR motifs, and actin.

MATERIALS AND METHODS

Phytophthora sojae Strain and Growth Conditions

P. sojae race R2 (isolate P6497) was grown and maintained on V8 agar (200 ml of V8 vegetable juice, 2.5 g of $CaCO_3$, and 15 g of bacto-style agar per liter of ddH_2O) plates at 20-25°C for 5-7 days in the dark. Prior to sub-culture of a new plug, a polycarbonate membrane filter was placed over the agar. Mycelium was scrapped from the membrane and crushed in liquid nitrogen with a mortal and pistol to a fine powder. Crushed mycelium was stored as 100 mg aliquots in microcentrifuge tubes at -80°C until further processing.

5-Aza-2′-Deoxycytidine Treatment

DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine purchased from Sigma-Aldrich was dissolved in 1 ml of a mixture of glacial acetic acid: water (1:9). 40 ml of V8 agar was treated with 100 μl of the dissolved 5-aza-2′-deoxycytidine [20 mM]. In one condition, *P. sojae* was grown in the dark and continually transferred to the treated agar for many subcultures (minimum of 25). Each subculture was allowed to grow for a period of 3-7 days. In a second condition *P. sojae* was exposed to 5-aza-2′-deoxycytidine for only one subculture (4 days). Mycelium was collected as described above.

Gene Candidates and Expression Data

Promoter regions of six genes, three genes containing Myb DNA-binding domains (*MYB),* two genes containing RXLR motifs, and actin were selected for analysis. Sequences of

these gene candidates were obtained from the Joint Genome Institute version 3.0 and the

VBI Microbial Database version 5.0. Candidate IDs are as follows: *Myb*554, *Myb*379,

*Myb*563, *Avr1a*, *Avr1b*, and actin; primers used to quantify transcripts are listed in table 2.

Electronic gene expression data was obtained from the VBI Microbial Database.

DNA Isolation

DNA extraction was adopted from the protocol of Zelaya-Molina *et al.*, (2010).

Modifications to the protocol were as follows: 1 µl of 100-mg/ml RNase A (Sigma-Aldrich,

MO), 800 µl digestion buffer, and 2 µl of 20-mg/ml proteinase K were added to 100-mg

frozen crushed mycelium and incubated at 37°C for 30 minutes. Following incubation,

samples were vortexed for 30 seconds and then angled in a shaker at 50 rpm for 30

minutes at 55°C. 800 µl of phenol:chloroform:isoamyl alcohol (35:24:1) was added to each

sample and vortexed until the two phases formed an emulsion. Samples were centrifuged

to separate the phases and the top layer of supernatant was transferred to a new 1.5-ml

microcentrifuge tube. The phenol:chloroform:isoamyl alcohol step was repeated using

equal amounts to the recovered sample. To precipitate DNA (the top layer of supernatant)

was transferred to a new microcentrifuge tube containing 700 µl of isopropanol. Tubes

were mixed by inversion and incubated at -20°C for 15 minutes. To pellet DNA, samples

were centrifuged at ~10,000×*g* for 10 minutes and the supernatant was discarded. The

pellet was rinsed a final time with 70% ethanol (vol/vol) and then immediately removed

by pipetting. Any remaining alcohol was evaporated using a heat block at 55°C for 5-10

minutes. DNA was resuspended in 100 µl of warm 10 mM Tris-HCl (pH 8).

RNA Isolation

Total RNA from control and 5-aza-2′-deoxycytidine treated *P. sojae* was extracted using the RNeasy Plant Mini Kit (QIAGEN, CA) following the procedures described by the manufacturer. RNA was eluted twice; each time in a clean microcentrifuge tube with 30 μl RNase-free water. The concentration of RNA was determined by NanoDrop-spectrophotometry and the quality was evaluated using agarose gel electrophoresis.

cDNA Synthesis

First strand cDNA was synthesized according to the manufacturer's protocol for Superscript III Reverse Transcriptase using oligo (dt)$_{18}$ primer (Invitrogen, CA). Samples were incubated for 5 minutes at 65°C, and then immediately placed on ice for 1 minute to prevent the formation of secondary structures. The following components were then added to the microcentrifuge tube: 5× first-strand buffer, 0.1M DTT, 40 units/μl RNaseOUT ribonuclease inhibitor, and 200 units/μl superscript reverse transcriptase III. Contents were mixed by pipetting and incubated for 45 minutes at 50°C. A final incubation period at 70°C for 15 minutes inactivated the reaction.

Methylated DNA Immunoprecipitation

DNA was sheared using Bioruptor sonicator (Diagenode Inc.) for 30 minutes. Sonication occurred in 30-second intervals with 30-second pauses in between, at high ultrasound wave output power. Ice was changed every 10 minutes. Fragmentation of DNA between 300- 600 bp was viewed on an agarose gel.

Immunoprecipitation was performed using the Methylated-DNA IP kit (Zymo Research, CA), according to the protocol described by the manufacturer with slight modifications. Input DNA was immunoprecipitated with either Mouse Anti-5-Methylcytosine monoclonal antibody (McAb) (Zymo Research, CA) or Mouse IgG (Invitrogen, CA) in a DNA:McAb ratio of 1:10. Alterations to the protocol are as follows: MIP buffer, 50% Protein A-Sepharose Slurry (Millipore, CA), and antibody were incubated on a rocker at 4°C for 30 minutes and then placed at room temperature for an addition 10 minutes. 400 ng of input DNA was diluted with DNA denaturing buffer to a final volume of 50 μl and denatured in a heat block at 98°C for 5 minutes. DNA samples were then immediately placed on ice. Denatured DNA was mixed with the antibody/protein A mixture and incubated at 37°C in a shaker at 50 rpm for a minimum of 2.5 hours. Washing of immune-complexes was carried out as described by the manufacturer. After the final rinsing of recovered DNA, an addition centrifuge step at 10,000×*g* for 1 minute was preformed to ensure the column did not contain excess buffer. DNA was eluted twice, each time in a clean microcentrifuge tube with 10 μl of DNA elution buffer provided by Zymo Research.

Polymerase Chain Reaction and Sequencing

Following immunoprecipitation, individual regions were identified by PCR and input DNA was used as a positive control. Upstream promoter regions were amplified using 2× Taq DNA polymerase Master Mix (NEB, MA). Primers designed to the promoter region are listed in Table 1. PCR conditions started with an initial denature step at 95°C for 2 minutes. This was followed by 25 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and

72°C for 30 seconds. The program ended with a final extension period at 72°C for 5 minutes. PCR samples were purified using Qiaquick PCR purification kit (QIAGEN, CA), following the procedure outlined by the manufacturer. The purified amplicons were sent for sequencing at the Cancer Research Center DNA Sequencing Facility in Chicago. Template concentrations were determined by NanoDrop-spectrophotometry and prepared according to the guidelines of the sequencing facility.

Table 1. Primers for methylated DNA immunoprecipitation.

Gene	Forward Primer, 5′ to 3′	Reverse Primer, 5′ to 3′	Expected Size
Myb554	CATTTCAGGGGCATTCATTC	GGTACATCGCGGAATCACTT	249 bp
Myb563	GCCAAACAATGCTCAAACCT	CAACGAATTGTGGAATGACG	173 bp
Myb379	CAGCTTTCAGCCACGTCTTC	AAACTACCCTTTGGCCGTTC	222 bp
Avr1a	TACAATCTGTGCGAAAAACGTC	AGACCACGATGACCTTGAATCT	210 bp
Avr1b	GAGTCTAGCGCAGCCATACC	GTCGTTCTGTTGCACTGGAA	223 bp
Actin	ACGGCGTTTCACTTGGTTTC	TGGACTTGGGAGTCTTGGAGA	220 bp

Quantitative Polymerase Chain Reaction

Quantitative PCR was used to determine the degree of non-specific binding during immunoprecipitation when IgG was used.. Target regions were amplified by Dynamo 2× qPCR master mix (Thermo Fisher Scientific, CA) containing SYBR green fluorescence in the Miniopticon (Bio-Rad, CA). For each target region, 3 replicate qPCR reactions were preformed using 15 ng of template per reaction from either IgG or anti-5-methylcytosine

immunoprecipitation samples. qPCR conditions are as follows: an initial denature at 95°C

for 3 minutes followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and an

extension period at 72°C for 30 seconds. A plate read was taken after each extension step.

The melt curve was set from 65°C to 90°C and a plate read was taken in .1°C increments,

holding each time for 3 seconds. CFX manager software (Bio-Rad, CA) was used to

analyze data. Primers used are listed in Table 1.

Gene Expression Analysis by qRT-PCR

To investigate expression data of candidate genes from untreated and 5-aza-2′-

deoxycytidine treated mycelium samples, qPCR was preformed as followed, RNA was

isolated and synthesized to first-strand cDNA as described above. The qPCR was

preformed in 15 μl reactions containing 0.83 ng of cDNA, 7.5 μl of 2× Dynamo SYBR

master mix, 1 μM of gene-specific primer, and 6.3 μl of distilled H_2O. The reactions were

preformed on a Miniopticon real-time PCR instrument (BIO-RAD, CA) under the

following conditions: 95°C for 3 minutes; 39 cycles of 95°C for 15 seconds, 52°C or 55°C for

15 seconds, and 72°C for 15 seconds. A plate read was taken after each extension step. The

melt curve was set from 65°C to 95°C and a plate read was taken in 1°C increments,

holding for 3 seconds each time. CFX manager software was used to obtain expression

level of each sample. Primers used are listed in Table 2.

Table 2. Primers used to quantify transcripts.

Gene	Forward Primer, 5′ to 3′	Reverse Primer, 5′ to 3′

Myb554	GACTTCATCGGCACCAAGAC	GGCGTGGCTACGTTACTGTT
Myb563	CAGACCCACACGCAAAAGTA	GCAGAACTGGAGGATGTCGT
Myb379	GACTTCCATGGCTGCTTCAT	GGCTGCGAAGTTGGAGAAC
Avr1a	CGATGTCCTCACCACTGAGA	GCTCGTTACCCTTCCATTTG
Avr1b	AGGAGCAGAAAGCGTACGAG	ACGGTCGTACTTCCCCTTCT
Actin	AGCGTATGACCAAGGAGCTG	TTCGAGATCCACATCTGCTG

5-aza-2′-deoxycytidine and DNA Damage

To examine DNA damage caused by 5-aza-2′-deoxycytidine, *P. sojae* mycelium was treated with the drug for 1 subculture (4 days) and DNA was isolated from treated mycelium as described above. Target regions were amplified by PCR using the same cycling conditions as previously described. All samples were purified with Qiaquick PCR purification kit (QIAGEN, CA) following the manufacturer's protocol. Samples were sent for sequencing at the University of Cincinnati and Children's Hospital DNA Core Facility.

Sequence Analysis

All sequences were manually verified compared to the chromatogram. The manually annotated sequences were analyzed by BLASTn through three different genome sources, the Broad Institute, the Joint Genome Institute, and the VBI Microbial Database. Sequences were aligned by ClustalW at EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Promoter sequences for all six-candidate genes were analyzed for CpG islands using the CpG island searcher (http://cpgislands.usc.edu). The search criteria was set at 55% GC

content with an observed CpG/expected CpG ratio of ≥0.65. 1,000 bps upstream to the

start site of each gene was obtained from JGI V3.0 and used in the analysis.

RESULTS

<u>Identification of CpG Islands in Promoter Regions</u>

Four out of six candidate genes contain a CpG island in the corresponding

promoter regions (Figure 2). *Myb*554 has one CpG island located at -1009 bp upstream

from the predicted transcription start site. This CpG island contains a total of 102 CpG

dinucleotides. *Myb*563 has one CpG island located between -1288 bp to -307 bp, with a

total of 84 CpG dinucleotides. *Myb*379 contains one CpG island located between -1000 bp

to -1 bp, with a total of 108 CpG dinucleotides. Actin (543739) also contained one CpG

island located between -707 bp to -1 bp with a total of 58 CpG dinucleotides. Surprisingly,

CpG islands were not detected in the promoter regions of the two genes encoding for

either effector protein *Avrla* or *Avr1b*.

A.

B.
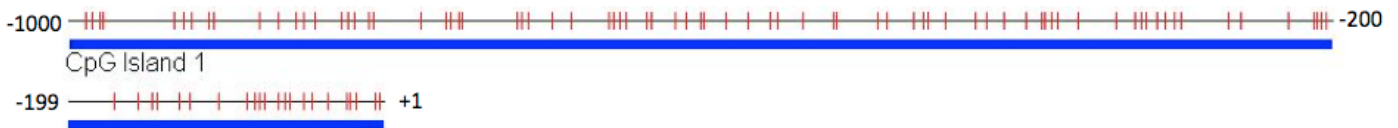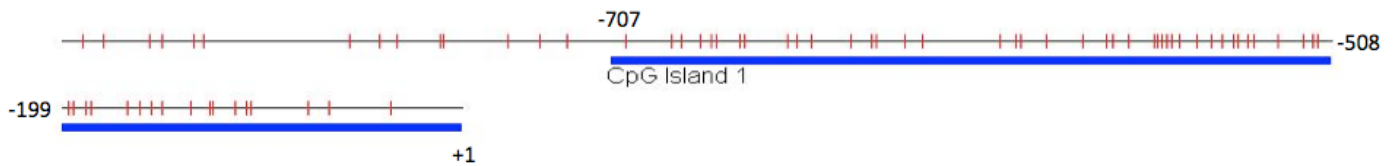

C.

D.

Figure 2. CpG islands in promoter regions: *Myb*554 (A), *Myb*563 (B), *Myb*379 (C), and

actin (D). Each vertical bar represents a CpG dinucleotide. The numbers indicates the

upstream position to the start codon (+1).

<u>Immunoprecipitation</u>

The MeDIP approach was utilized to evaluate the methylation status of *P. sojae*. 5-methylcytosine was detected in the promoter regions, predicted *in silico*, of the six-candidate genes mentioned (Figure 3). Targeted regions obtained by PCR were sent for sequencing to ensure primers were binding to the correct locations. Sequence analysis by BLASTn through the genome websites, Broad Institute, JGI, and VMD, identified all the six promoter regions as expected (Figure 4). To account for nonspecific binding of the anti-5-methylcytosine antibody, qPCR was used to compare the relative amount of DNA immunoprecipitated with IgG. The results indicate a very low level of nonspecific binding (Figure 5). To test the efficiency of MeDIP, samples were spiked with 1 ng of methylated control DNA provided by Zymo Research, CA (data not shown).
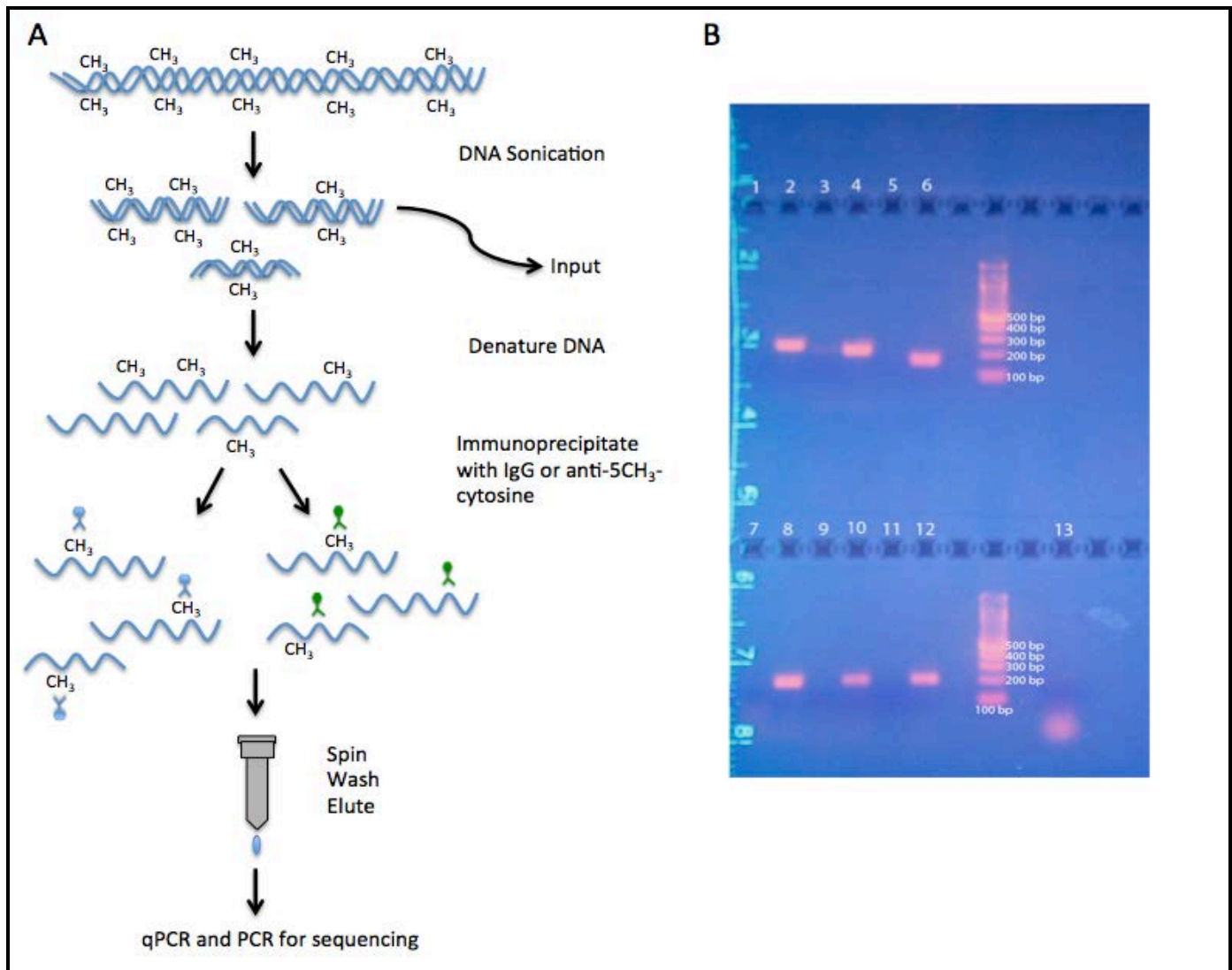
Figure 3. MeDIP: Experimental outline of immunoprecipitation (A). Agarose gel electrophoresis of promoter regions of candidate genes amplified by PCR from the immunoprecipitated pool (B). Lane 1 *Myb*554 immunoprecipitated with IgG. Lane 2 *Myb*554 immunoprecipitated with 5-methylcytosine antibody. Lane 3 *Myb*379 with IgG. Lane 4 *Myb*379 with anti-5-methylcytosine. Lane 5 *Myb*563 with IgG. Lane 6 *Myb*563 with anti-5-methylcytosine. Lane 7 *Avrla* with IgG. Lane 8 *Avrla* with anti-5-methyltyctosine.

Lane 9 *Avrlb* with IgG. Lane 10 *Avrlb* with anti-5-methylcytosine. Lane 11 actin with IgG.

Lane 12 actin with anti-5-methylcytosine. Lane 13 is negative control.

A.

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MeDIPActin    AGGACGGCTGACGTGCACCATTACACTCCCGCTCTCTATCTCGCGGGAGGGAAGACCCGC 60
RefActin      AGGACGGCTGACGTGCACCATTACACTCCCGCTCTCTATCTCGCGGGAGGGAAGACCCGC 60
              ************************************************************

MeDIPActin    CTGTCGTCGCTTCACCTCAGAGCCAACTCCAACTCCCCCTCTTCGAAGTGGATCCACGTG 120
RefActin      CTGTCGTCGCTTCACCTCAGAGCCAACTCCAACTCCCCCTCTTCGAAGTGGATCCACGTG 120
              ************************************************************

MeDIPActin    GCCTGGGAGGCTCATTCCCCATTTCCCCCAGTTGGCGGCAGATCTCCAAGACTCCCAAGT 180
RefActin      GCCTGGGAGGCTCATTCCCCATTTCCCCCAGTTGGCGGCAGATCTCCAAGACTCCCAAGT 180
              ************************************************************

MeDIPActin    CCAA- 184
RefActin      CCACT 185
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B.

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MeDIPMyb554   GATTCCGTCAACCCATCGCGCTTTTTGTCCAATAAGAAAACACATAAAATGGGCTTCGTG 60
RefMyb554     GATTCCGTCAACCCATCGCGCTTTTTGTCCAATAAGAAAACACATAAAATGGGCTTCGTG 60
              ************************************************************

MeDIPMyb554   CCAAAACCAGGTACCAACTACCAGCTCAACTTTGGGGGACCGGTGCCAGAACCCGTTGCT 120
RefMyb554     CCAAAACCAGGTACCAACTACCAGCTCAACTTTGGGGGACCGGTGCCAGAACCCGTTGCT 120
              ************************************************************

MeDIPMyb554   CGCAGTGCGAAAACCCGTCGCAAGCGCGACCGCGACGTCGGACCAGTATACAAACGCCAA 180
RefMyb554     CGCAGTGCGAAAACCCGTCGCAAGCGCGACCGCGACGTCGGACCAGTATACAAACGCCAA 180
              ************************************************************

MeDIPMyb554   GTGATTCCGCGATGTACCA- 199
RefMyb554     GTGATTCCGCGATGTACCCA 200
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C.

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MeDIPAvr1a    --TACTGAAACACATATTATTTCTTATGCTGTCCGGGACGGTTTAAGCGTTTTAAATGAC 58
RefAvr1a      TATACTGAAACACATATTATTCCTTATGCTGTCTGGGACGGTTTAAGCGTTTTAAATGAC 60
              ****************** ********** *************************

MeDIPAvr1a    CTCTCCGCGTCCAACGGGTTAATAGACATGTACAGTATATGTATTACCGTCACTTGACGA 118
RefAvr1a      CTCTCCGCGTCCAACGGGTTAATAGACATGTACAGTATATGTATTACCGTCACTTGACGA 120
              ************************************************************

MeDIPAvr1a    ATCACACTGTGACGGTCTCCAGACCACGATGACCTTGAATCTA 161
RefAvr1a      ATCACACTGTGACGGTCTCCAGACCACGATGACCTTGAATCTC 163
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Figure 4. MeDIP sequence alignments: Target promoter regions pulled down by MeDIP

were aligned against reference sequences from the genome database using ClustalW

(http://www.ebi.ac.uk/Tools/msa/clustalw2/). These examples show the alignment of

actin (A), *Myb*55 (B), and *Avr1a* (C).
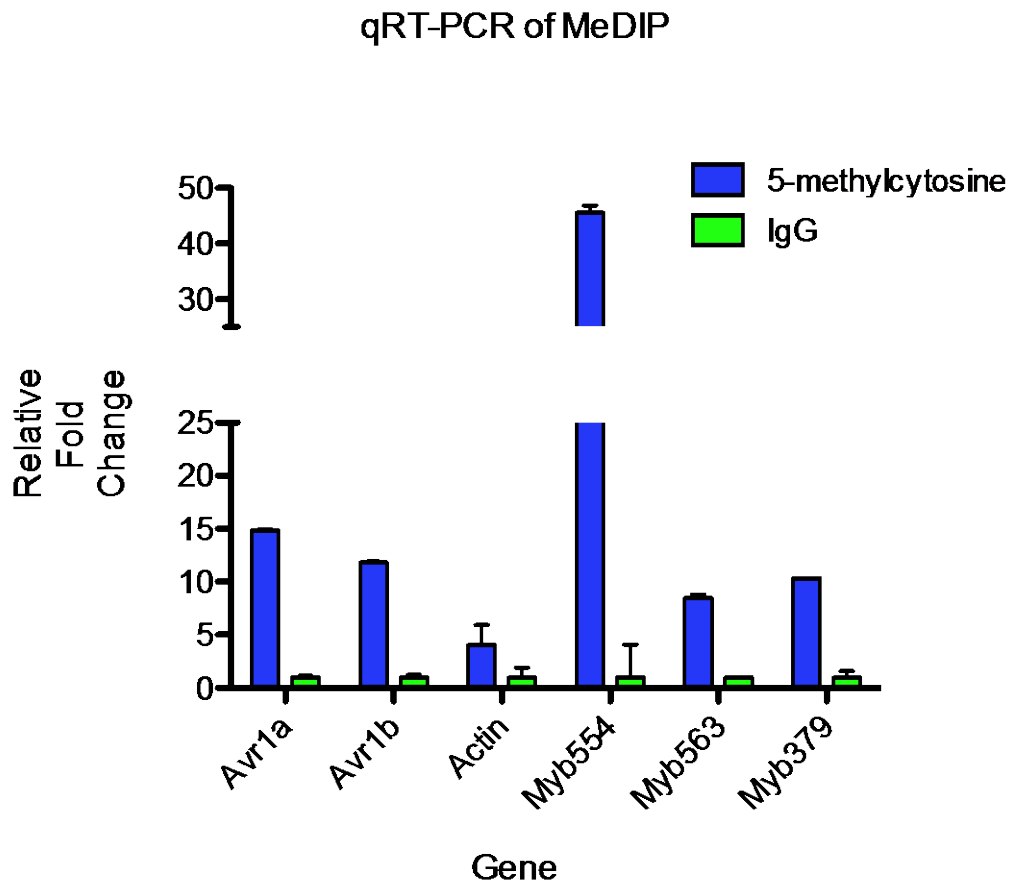
qRT-PCR of MeDIP

Figure 5. qPCR analysis of promoter regions pulled down from MeDIP with either 5-methylcytosine antibody or IgG: Relative quantitative change for each target was calculated from average cycle threshold values (C_T) where quantitative change = $2^{(C_T(IgG) - C_T(5-CH_3))}$. IgG antibody was used as a control and set at 1; error bars correspond to one standard deviation.

5-aza-2′-deoxycytidine and Gene Expression

Previous findings show that incorporation of 5-aza-2′-deoxycytidine (5-azadC) inhibits DNA methyltransferases therefore leading to a loss of methylation. Consequently, silenced genes induced by DNA hypermethylation are reactivated after treatment (Zhu *et al*, 2004). To determine the effects of methylation on gene expression in *P. sojae,* mycelium was exposed to 5-azadC [20 mM] for approximately 25 subcultures on V8 agar plates and then the hyphal mat was collected for expression analysis by qRT-PCR. *P. sojae* displayed an insignificant decreased growth phenotype in the presence of 5-azadC for each subculture (data not shown). However, when the hyphal plug was transferred onto fresh-made V8 media containing 5-azadC after approximately 25 subcultures, *P. sojae* stopped growing indicating that 5-azadC eventually killed *P. sojae.* These findings correlate with previous studies that have shown 5-aza-2′-deoxycytidine treatment is perceived as DNA damage and leads to cell cycle arrest in the G1 phase through the p53/p21[WAF1/CIP1] pathway (Zhu *et al*, 2004). Gene expression data by qRT-PCR shows decreased gene expression of all genes tested in 5-azadC samples (Figure 6). This is most likely due to the decreased growth rate resulting in fewer transcripts caused by DNA damage rather than the effects of methylation at these sites.

Due to the cytotoxicity and mutagenic potential of 5-aza-2′-deoxycytidine, the duration of treatment was reduced to 1 subculture. *P. sojae* control and 5-azadC [20 mM] samples were transferred at the same time and incubated in the dark for a period of 4 days. The hyphal mat was collected for gene expression data. Expression levels of all

genes tested in control samples were higher compared to 5-azadC treated samples (Figure 7). *P. sojae* still displayed a slight decreased growth phenotype in the presence of the drug at the concentration used in our experiment, suggesting a toxic effect on growth and development of *P. sojae.* Previous work by Zhu et al. (2004) has shown a dose-dependent inhibition of cell proliferation caused by 5-aza-2′-deoxycytidine. When human lung cancer cells were treated with the drug for a period of 72 hours at different concentrations, cell viability was decreased to 77% of the untreated control even at a very low dose of 5-aza-2′-deoxycytidine [.078 μM]. At concentration of 1.25 or 5 μM, cell viability decreased to 51% or 39%, respectively.

Incorporation of 5-azadC occurs during DNA replication and binds DNA methyltransferases in an irreversible manner (Santi *et al,* 1984; Christman, 2002). This creates methyltransferase-5azadC adducts and depletion of methyltransferase protein levels (Palii *et al*, 2008). It has been shown that 5-azadC-methyltransferase protein crosslinks are mutagenic if not repaired (Jackson-Grusby *et al*, 1997). In this study, promoter regions of the same set of genes exposed to 5-azadC for 1 subculture were amplified by PCR, purified and sent for sequencing to analyze for DNA damage. Sequencing results showed base deletions and single nucleotide polymorphisms (SNPs) in 5-azadC treated samples. There were 8 SNPs in the promoter region of *Avr1b* compared to control samples at positions -186, -185, -182, -178, -172, -171, -167, and -149 upstream to the start codon. Myb554 showed 8 base deletions at positions -312, -300, -285, -270, -262, -261, -251, and -239; as well as one SNP at position -298. Actin had one base deletion at position -

177 upstream to the start codon (Figure 8). Similarly, Jackson-Grusby et al. (1997)

sequenced mutations in a reporter *Escherichia coli lac I* transgene isolated from colonic

DNA from mice with and without 5-azadC administration and showed that mutations

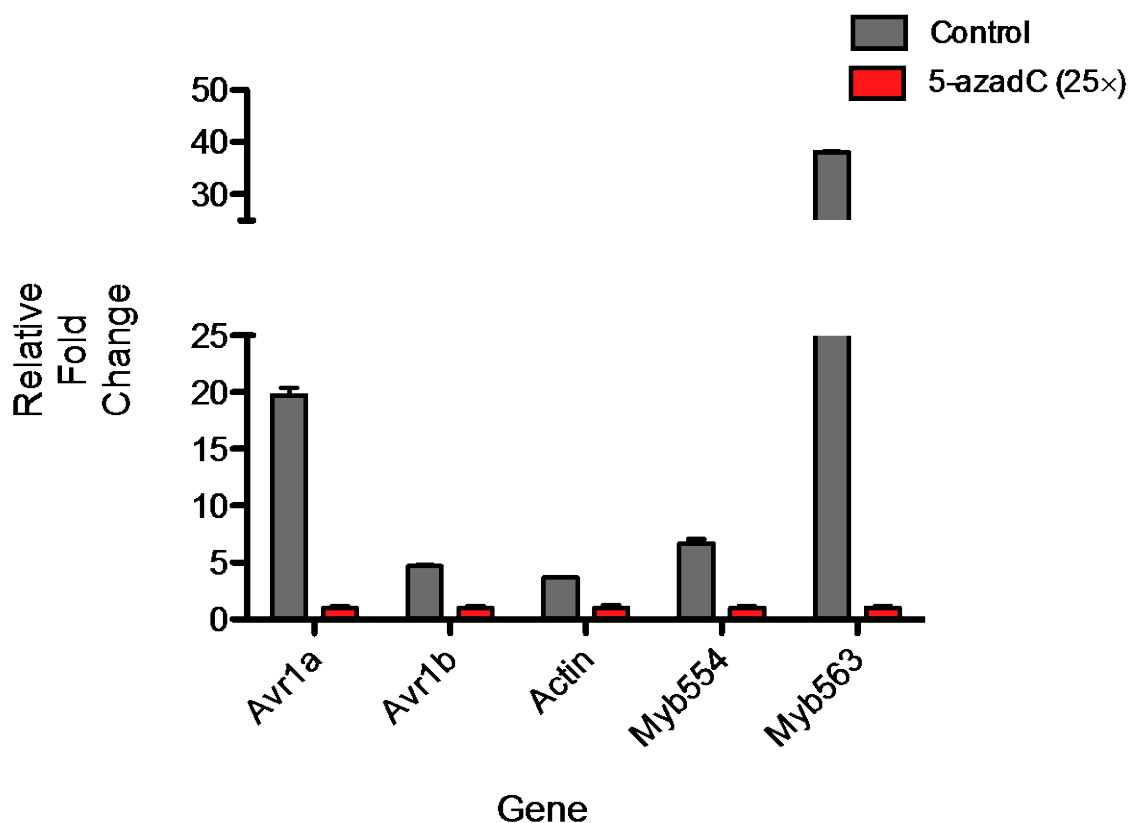induced by 5-azadC predominately occurred at CpG dinucleotides.

Figure 6. Expression analysis by qRT-PCR of *P. sojae* hyphae grown in the presence or absence of 5-aza-2′-deoxycytidine (5-azadC) treated samples for approximately 25 subcultures: The low level of expression was observed in the 5-azadC samples, which was set at 1. Relative fold expression for each target was calculated from average cycle

threshold values (C_T) where fold expression = $2^{(C_T(\text{5-azadC}) - C_T(\text{Control}))}$. Error bars correspond to one standard deviation. For example, Myb554 control has 6.68× more transcripts than Myb554 5-azadC.
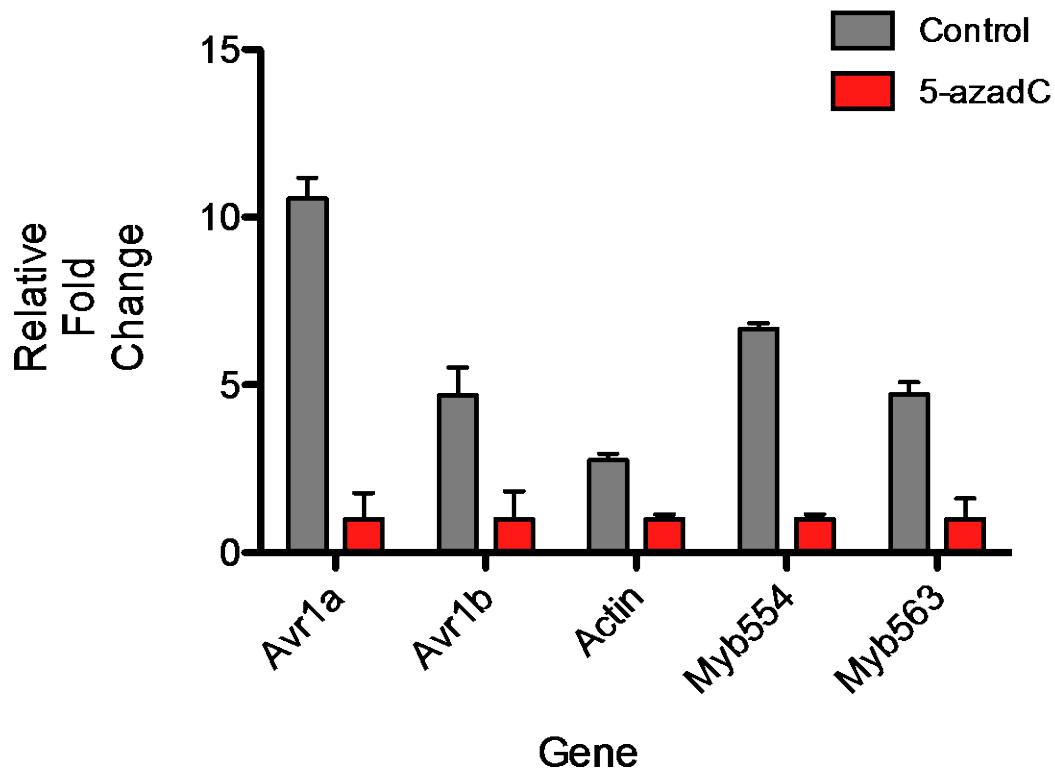

Figure 7. Expression data by qRT-PCR of *P. sojae* grown on V8 agar in the presence or absence of 5-azadC for one subculture: Relative fold expression was calculated as previously described and expression of 5-azadC was set at 1. Error bars correspond to one standard deviation.

A.

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Myb554_Control    -CACCCATCGCGCTTTTTGTCCAATAAGAAAACACATAAAATGGGCTTCGTGCCAAAACC 59
Myb554_Control2   CAACCCATCGCGCTTTTTGTCCAATAAGAAAACACATAAAATGGGCTTCGTGCCAAAACC 60
Myb554_5azadC     -CACCC-TCGCGCTTTTT-TGCAATAAGAAAAC-CATAAAATGGGCTTC-TGCCAAA--C 53
                   .**** ********** * *********** *************** ******* *

Myb554_Control    AGGTACCAACTACCAGCTCAACTTTGGGGGACCGGTGCCAGAACCCGTTGCTCGCAGTGC 119
Myb554_Control2   AGGTACCAACTACCAGCTCAACTTTGGGGGACCGGTGCCAGAACCCGTTGCTCGCAGTGC 120
Myb554_5azadC     AGGTACCA-CTACCAGCTCA-CTTTGGGGGACCGGTGCCAGAACCCGTTGCTCGCAGTGC 111
                  ******** ********** ****************************************

Myb554_Control    GAAAACCCGTCGCAAGCGCGACCGCGACGTCGGACCAGTATACAAACGCCAAGTGATTCC 179
Myb554_Control2   GAAAACCCGTCGCAAGCGCGACCGCGACGTCGGACCAGTATACAAACGCCAAGTGATTCC 180
Myb554_5azadC     GAAAACCCGTCGCAAGCGCGACCGCGACGTCGGACCAGTATACAAACGCCAAGTGATTCC 171
                  ************************************************************

Myb554_Control    GCGATGTACCA 190
Myb554_Control2   GCGATGTACCA 191
Myb554_5azadC     GCGATGTACC- 181
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B.

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Actin_5azadC      --GACGGCTGACGTGCACCATTAC-CTCCCGCTCTCTATCTCGCGGGAGGGAAGACCCGC 57
Actin_Control2    AGGACGGCTGACGTGCACCATTACACTCCCGCTCTCTATCTCGCGGGAGGGAAGACCCGC 60
Actin_Control     -GGACGGCTGACGTGCACCTTTACACTCCCGCTCTCTATCTCGCGGGAGGGAAGACCCGC 59
                    ****************:**** ***********************************

Actin_5azadC      CTGTCGTCGCTTCACCTCAGAGCCAACTCCAACTCCCCCTCTTCGAAGTGGATCCACGTG 117
Actin_Control2    CTGTCGTCGCTTCACCTCAGAGCCAACTCCAACTCCCCCTCTTCGAAGTGGATCCACGTG 120
Actin_Control     CTGTCGTCGCTTCACCTCAGAGCCAACTCCAACTCCCCCTCTTCGAAGTGGATCCACGTG 119
                  ************************************************************

Actin_5azadC      GCCTGGGAGGCTCATTCCCCATTTCCCCCAGTTGGCGGCAGATCTCCAAGACTCCCAAGT 177
Actin_Control2    GCCTGGGAGGCTCATTCCCCATTTCCCCCAGTTGGCGGCAGATCTCCAAGACTCCCAAGT 180
Actin_Control     GCCTGGGAGGCTCATTCCCCATTTCCCCCAGTTGGCGGCAGATCTCCAAGACTCCCAAGT 179
                  ************************************************************

Actin_5azadC      CCAA 181
Actin_Control2    CCAA 184
Actin_Control     CCAA 183
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C.

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Avr1b_5azadC      GATACATTATCGAAATGAAATGTTCATTTCGTCTGTTTCACGCCATCACTGATTCTTGTC 60
Avr1b_Control     GATACATTATCGAAATGAAATGTTCATTTCGTCTGTTTCACGCCATCACTGATTCTTGTC 60
                  ************************************************************

Avr1b_5azadC      GATCAGAATCAAACACCGAGGAGGTTGCCTTCGAAAAAAAAAAAACAATAATACACCCCA 120
Avr1b_Control     GATCAGAATCAAACACCGAGGAGGTTGCCTTCGAAAAAAAAAAAACAATAATACAATCCA 120
                  *********************************************************. ***

Avr1b_5azadC      GAGCGTGCACCCCCAGGGAAATGATCCCTTGTCTTTCTGTTGCA--- 164
Avr1b_Control     AAGCATGCACATCCAAGGAAATGATCCCTTGTCGTTCTGTTGCACTG 167
                  .***.*****. ***.**************** **********
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Figure 8. Sequence alignments of control and 5-azadC treated samples for one

subculture using clustalW: These examples show DNA damage caused by 5-azadC in

Myb554 (A), actin (B), and Avr1b (C). Two control sequences were used to preform the alignment; control represents untreated mycelium and control2 represents sequences pulled down by MeDIP.

DISCUSSION

DNA methylation is a major epigenetic control where changes in gene expression occur without changes to the DNA sequence. This epigenetic modification is widely distributed in both prokaryotic and eukaryotic organisms. Eukaryotic methylation is involved in many biological processes such as DNA replication, gene regulation, chromatin structure, and transposition. Transcriptional gene silencing (TGS) in plants and animals is associated with altered methylation patterns and chromatin structure. Evidence of gene silencing at the transcriptional and post-transcriptional level has previously been shown to operate in *P. infestans* (van West *et al*, 1999; van West *et al*, 2007). Digestion of DNA from silenced-*inf1* transgenic strains with methylation sensitive restriction endonucleases and sequencing of bisulfite-treated DNA did not show any evidence of DNA methylation at the silenced loci (van West *et al*, 2007).

Despite the lack of detectable cytosine methylation, 5-azacytosine, a methyltransferase inhibitor, could reverse silencing in *P. infestans*. It is possible that 5-azacytosine could inhibit other methyltransferases such as histone methyltransferases, which could explain reversal of silencing after treatment with this chemical (Wozniak *et al*, 2006). *Inf1*-silenced transformants were also treated with the histone deacetylase inhibitor trichostatin A that also led to a reversal of silencing. From this, it has been speculated that TGS in *P. infestans* and other oomycetes is not based on DNA-methylation but histone deacetylation and methylated heterochromatin (Whisson *et al*, 2009). However, these

findings do not rule out the possibility of DNA methylation because only 1 locus was investigated.

For a long period of time, other organisms such as yeast, *C. elegans*, and *Drosophila* have been reported to lack detectable methylation of their genomes (Lyko, 2001). However, it was discovered that during early embryonic development of *Drosophila,* methylated cytosine bases are present (Lyko, 2000). The fruit fly also shows unusual sequence specificity of DNA methylation. Only a small fraction of 5-methylcytosine was found at CpG dinucleotides; most of the 5-methylcytosine occurred in the context of CpT and CpA dinucleotides (Lyko, 2000).

The goal of this work was to investigate the presence of DNA methylation in the oomycete *P. sojae*. Results from MeDIP indicated that cytosine methylation does occur in the genome of *P. sojae*. Methylation was detected in the promoter region of a range of genes, which suggest that *P. sojae* also employed DNA methylation as one of the mechanisms of gene regulation. Interestingly, methylation was detected in the promoter regions of two avirulence genes, *Avr1a* and *Avr1b.* Among different races of *P. sojae,* these effectors display variation in expression and DNA methylation could be the mechanism used to regulate the expression of these genes.

Four out of the six candidate genes pulled-down by MeDIP were predicted to contain a CpG island in the corresponding promoter regions, while CpA is the most frequent dinucleotide present in the promoter region of the other two genes. Digestion of *P. sojae* genomic DNA with cytosine methylation sensitive enzymes did not produce any

bands shifts, suggesting cytosine methylation in *P. sojae* may differ from other eukaryotes (data not shown).

The role cytosine methylation plays within the oomycete is unclear. Addition of 5-azadC to the growth medium resulted in a slightly decreased growth phenotype, therefore less transcripts were detected in the qRT-PCR analysis. The decreased expression is mostly likely due to DNA damage caused by 5-azadC rather than effects of methylation. A dose-dependent assay of 5-azadC below the DNA damage concentrations could be used to clarify if methylation in *P. sojae* is involved in gene regulation.

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