The Epigenetics of a Cereal Killer: The Role of DNA Methylation in Pathogenicity and Development of *Fusarium graminearum*

By

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Abstract

Epigenetics is an emerging field in the regulation of biological processes in fungi. This study examines the role of one such epigenetic mechanism, DNA methylation, in development and pathogenicity of the fungal phytopathogen *Fusarium graminearum*. Two DNA methyltransferase genes were identified, and disruption of these genes resulted in growth, reproduction and pathogenicity related phenotypes. Deletion mutants displayed changes in growth, and were impaired in their ability to produce ascospores and infect susceptible wheat. Secondary metabolism was also affected in these strains, including deoxynivalenol toxin production, pigmentation, and the expression of novel unknown metabolites. Bisulfite sequencing resulted in the first DNA methylome of any *Fusarium* species, and analysis indicated DNA methylation is present on ~0.1% of genomic cytosines, consistent with other related fungi. Methylation sites were identified in genic regions of genes related to a variety of cellular processes including development and pathogenicity. Altogether, our analysis shed light on the important epigenetic process of DNA methylation in a fungal plant pathogen.

Dedicated to Chelsey K. Blackman
My motivation, my best friend and my biggest supporter

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List of Abbreviations

5mC – 5-Methyl Cytosine

6mA – N-6 Methyl Adenine

ACP – Acyl Carrier Protein

ADON – Acetyl-deoxynivalenol

AT – Acyltransferase

BER – Base Excision Repair

BLAST – Basic Local Alignment Search Tool

BR – Biological Replicate

CAGEF – Centre for the Analysis of Genome Evolution and Function

ChIP/ChIPseq – Chromatin Immunoprecipitation / sequencing

CK – Cytokinin

CMC – Carboxymethylcellulose

DIM – Deficient in Methylation

DMAP1 – DNMT1 Associated Protein

DMC – Differentially Methylated Cytosine

DMR – Differentially Methylated Region

DMT – DNA Methyltransferase

DON – Deoxynivalenol

ELISA – Enzyme Linked Immunosorbent Assay

FG1 – *Fusarium graminearum* DAOM241165

FG2 – *Fusarium graminearum* DAOM233423

FHB – Fusarium Head Blight

FPP – Farnesyl Pyrophosphate

GGPP – Geranyl Geranyl Pyrophosphate

GPP – Geranyl Pyrophosphate

HDAC2 – Histone Deacetylase

HOTAIR – *Hox* transcript antisense RNA

HPLC – High Performance Liquid Chromatography

HRM – High Resolution Melt

KS – Ketoacyl CoA Synthase

lncRNA – Long Non-Coding RNA

MBD – Methyl-Binding Protein

MIP – Methylation Induced Pre-meiotically

MIPS – Munich Information Centre for Protein Sequences

miRNA – Micro RNA

ML – Maximum Likelihood

MREs – Methylation Sensitive Restriction Enzymes

mRNA – Messenger RNA

MS – Mass Spectroscopy

MSP – Methylation Specific PCR

NCBI – National Center for Biotechnology Information

NIH – National Institute of Health

NIV – Nivalenol

NP – Nutrient Poor

NR – Nutrient Rich

NRPS – Non-ribosomal Peptide Synthases

piRNA – Piwi-Interacting RNA

PKS – Polyketide Synthase

PTGS – Post-Transcriptional Gene Silencing

PTM – Post Translational Modification

RT-qPCR – Quantitative Real Time PCR

RID – Repeat Induced Deficient

RIP – Repeat Induced Point Mutation

ROS – Reactive Oxygen Species

rRNA – Ribosomal RNA

siRNA – Small Interfering RNA

SM – Secondary Metabolite

sncRNA – Small Non-Coding RNA

snoRNA – Small Nucleolar RNA

TdT – Terminal Deoxynucleotidyl Transferase

TE – Transposable Element

TET – Ten-eleven Translocation

TRI – Trichothecene

tRNA – Transfer RNA

USER – Uracil-Specific Excision Reagent

UTR – Untranslated Region

WGBS – Whole Genome Bisulfite Sequencing

ZEN – Zearalenone

Chapter 1: *Fusarium graminearum*

1.1 *Fusarium graminearum*: General Overview

F. graminearum is one of the more important fungal pathogens that affect agriculture. It has a broad host range among the economically important small grain cereal crops and causes fusarium head blight (FHB), among other crop diseases. *F. graminearum* is responsible for millions of lost dollars annually with estimated losses due to FHB alone reaching $7.67 billion USD between 1993 and 2001 in wheat and barley cultivation (Nganje *et al.* 2004). Losses are in part due to poor yield caused by the disease, but also due to contamination of grains with mycotoxins produced by the fungus during infection that reduces the value of the grains. Regulation of toxin amounts, such as deoxynivalenol (DON), in contaminated grains is vital as toxins are harmful to human health at 1.0 μg kg^{-1} body weight (van der Lee 2015). As such, *F. graminearum* remains one of the most important and intensely studied plant pathogens.

F. graminearum is in the subphylum of Ascomycota and the order Hypocreales (Kendrick 2000). *F. graminearum* is prevalent worldwide, with various subspecies and chemotypes (Starkey *et al.* 2007). Chemotype is defined as isolates that produce alternate derivatives of DON, such as 15-acetyl DON (15-ADON) or 3-acetyl DON (3-ADON). In western Canadian provinces, the *F. graminearum* population consists mainly of 15-ADON chemotype, while in eastern Canadian provinces 3-ADON chemotypes dominate (Ward *et al.* 2008). Notably, the 3-ADON producing chemotypes are increasing in frequency in the western provinces, suggesting an ongoing shift in population dynamics

of this pathogen (Ward *et al.* 2008). A novel mycotoxin type, NX2, has also recently been discovered in North America; NX2 chemotypes have been identified in southern Ontario and Manitoba (Kelly *et al*. 2016).

1.1.1 *F. graminearum* Lifecycle

The complete life cycle of *F. graminearum* is well documented; it is homothallic such that it can generate progenies without outcrossing to other sexually distinct individuals (Trail 2009). The fungus overwinters on infected crop residues as saprophytic mycelia before warming conditions and favourable moisture levels result in the formation of both asexual condia and sexual ascospores (Goswami and Kistler 2004). The germination of sexual and asexual spores is favoured by relative humidity of >53% and >80%, respectively, and at temperatures between 28°C-32°C (Beyer *et al.* 2005; Gilbert and Tekauz 2000). Conidia are spread by the wind or by rain splash, while the sexual ascospores are forcibly discharged from the mature perithecia (Goswami and Kistler 2004). The highest documented launch speed at over 34 m s^{-1} enables ascospores to cover large distances by entering wind currents (Trail *et al*. 2005; Keller *et al.* 2014). Additionally, the discharge of ascospores is timed concurrent with the flowering of various host crops to facilitate the infection process (Goswami and Kistler 2004).

1.1.2 Pathology and Infection

F. graminearum is a pathogen of a number of cereal crops, such as *Triticum* (e.g. wheat), *Hordeum* (e.g. barley), *Oryza* (e.g. rice), *Zea* (e.g. corn) and *Avena* (oats) among others (Goswami and Kistler 2004). It is the main causal agent of FHB, seedling blight,

brown foot rot and crown rot, to name a few (Goswami and Kistler 2004; Charkraborty *et al.* 2010). Infection of host crops begins with fungal spores deposited on inflorescence tissues, followed by the development of hyphae on the exterior surfaces of the florets and glumes. Hyphal growth is then directed towards open stomata or other exposed areas present on the host (Bushnell and Leonard 2003). Recent studies have observed infection structures, such as compound appressoria and infection cushions that enable the fungus to directly penetrate into the glumes of wheat (Boenisch and Schafer 2011). Once inside the host, the fungus will spread through vascular bundles in the rachis, which block water and nutrient supply resulting in bleaching of the inflorescence tissues (Jansen *et al.* 2005). Common symptoms of FHB in wheat include dark necrotic tissue ('scab') on the exterior surfaces of the spikelet as well as browning or bleaching ('blight') (Goswami and Kistler 2004).

Infection of the host also includes the production of secondary metabolites, which facilitate the colonisation process. Some of these metabolites are mycotoxins such as DON and its derivatives. These compounds bind to the large ribosomal subunit and inhibit peptidyltransferase activity (Yazar and Omurtag 2008; Foroud *et al.* 2016). Upon penetrating the host, *F. graminearum* produces DON within the nodes and rachis in wheat and the toxin is required to overcome host defences (Ilgen *et al.* 2009). Since DON and its derivatives play an important role in the infection process, it is a subject of studies in many laboratories.

1.1.3 Secondary Metabolism

The fungal kingdom produces a wide variety of secondary metabolites (SMs), ranging from useful antibiotics to harmful mycotoxins (Zain 2011; Brakhage 2013). SMs are typically low molecular weight, bioactive compounds. These compounds are usually not considered essential for life, although they may confer a fitness advantage over other organisms (Pusztahelyi *et al.* 2015). They are products of primary metabolite precursors, such as acetyl-CoA and shikimate pathway derivatives, and display enormous chemical and structural diversity (Pusztahelyi *et al.* 2015; Nielsen and Nielsen 2017). The four major classes of fungal SMs include alkaloids, polyketides, non-ribosomal peptide and terpenes (Keller *et al.* 2005). Alkaloids are primarily derived from the amino acid tryptophan and typically contain nitrogen; some of the recognizable alkaloid products include ergot alkaloids such as ergotamine (**Figure 1A**; Keller *et al.* 2005). Polyketides are the most abundant secondary metabolites found in fungi (Keller *et al.* 2005). The aflatoxin produced by *Aspergillus sp.* is a product of polyketide synthases (PKS), as well as fungal pigments such as those from *Monascus* (**Figure 1B**; Yu *et al.* 2004; Feng *et al.* 2012). These specialized metabolites are synthesized by type I PKSs, a multifunctional protein with ketoacyl-CoA synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains (Keller *et al.* 2005). Precursor acyl-CoA molecules from primary metabolism, such as acetyl-CoA and malonyl-CoA, are condensed into carbon chains of varying length. Chain length as well as the number of reduction steps determines the diversity of polyketides (Keller *et al.* 2005). The non-ribosomal peptides SMs are derived from amino acids by large multi-domain enzymes known as non-ribosomal peptide synthases, or NRPSs (Wilkinson and Micklefield 2009). These large enzymes contain

domains, namely: an adenylation or A domain, a P domain (peptidyl carrier), a C domain (peptide bond formation), and a TE (thioesterase) domain (Keller *et al.* 2005). Diversity arises from variation in the number of domains in the NRPS as well as the length of peptide being produced (Keller *et al.* 2005). Common examples of NRPS derived peptides include cyclosporin, an immunosuppressive drug used following surgery, and ferricrocin, a siderophore responsible for solubilizing iron for uptake into cells (Weber *et al.* 1994; Eisendle *et al.* 2003). One of the more well-known NRPS derived peptide products is the β-lactam antibiotic, penicillin (**Figure 1C**; Smith *et al.* 1990). Penicillin and similar NRPS-derived metabolites highlight the importance of SMs as desirable products with large impacts on society (Keller *et al.* 2005). All terpenes are composed of isoprene units that can be further classified by the number of isoprene units in their backbone (Keller *et al.* 2005). Monoterpenes are synthesized from geranyl pyrophosphate (GPP), sesquiterpenes from farnesyl diphosphate (FPP), and multi-terpenes from geranyl-geranyl pyrophosphate (GGPP; Keller *et al.* 2005). Common fungal terpenes include the tetra-terpene carotenoids (fungal pigments), and the sesquiterpene trichothecene toxins such as DON (**Figure 1D**).

Individual fitness is the primary driving force for producing SMs by fungi, reflected by diversity of SMs involved in many fungi-microbe and fungi-plant interactions (Netzker *et al*. 2015; Rohlfs 2015; Pusztahelyi *et al*. 2015).

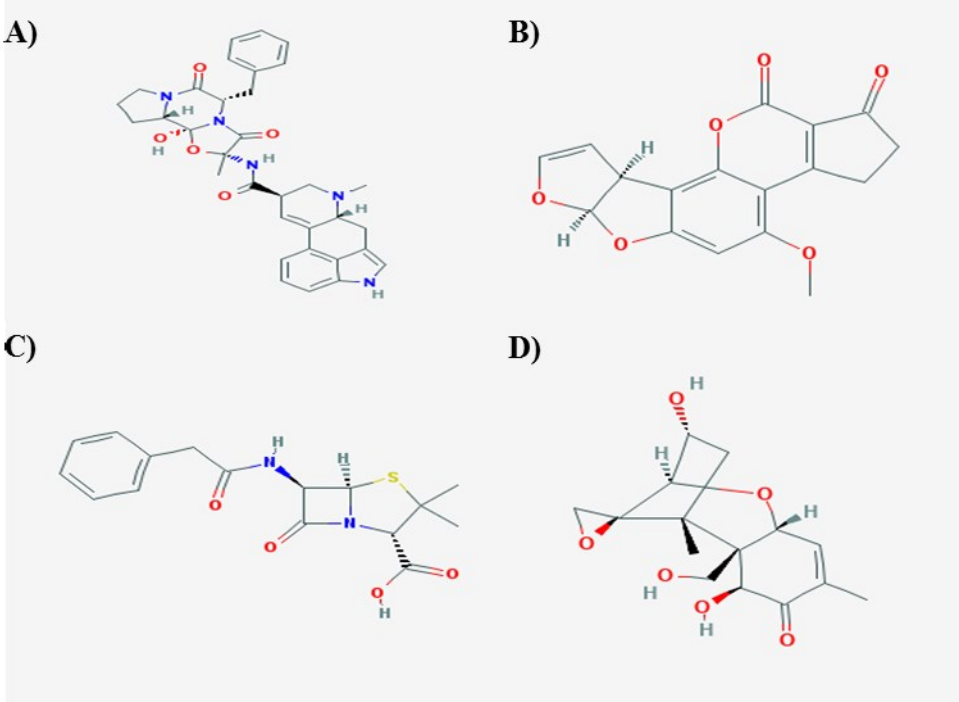


Figure 1: Example compounds of four major classes of fungal secondary metabolism. A) The alkaloid ergotamine. B) Polyketide synthase product aflatoxin. C) Non-ribosomal peptide synthase product penicillin. D) Terpene deoxynivalenol.

During colonization of host plants, pathogenic fungi utilize their arsenal of SMs in numerous ways to overcome host defences and thus increase individual fitness. Examples of these SMs include the production of a plant hormone cytokinin by the rice fungus *Magnaporthe oryzae* resulting in dampening of host defences as well as altering sugar and amino acid distribution in rice, and *F. graminearum*, which produces the protein synthesis inhibitor sesquiterpene DON (Chanclud *et al.*2016; Yazar and Omurtag 2008).

F. graminearum with 67 predicted secondary metabolite gene clusters is capable of producing numerous specialised metabolites (Sieber *et al.* 2014). A majority (54) do not correspond to any known product, but, genes from 20 of the clusters are induced *in planta* (Sieber *et al.* 2014). The 13 SM clusters with known products include the following mycotoxins: zearalenone, fusarin C, culmorin, the trichothecenes DON and NIV, as well as pigments such as aurofusarin (Desjardins 2006). Aurofusarin is highly conserved across *Fusarium* species, resulting in a red pigmentation in the fungal hyphae (Frandsen *et al.* 2006). The trichothecene (TRI) cluster is the most characterized SM cluster, and produces a variety of toxins such as T-2, NIV and DON and these have been strongly implicated in human and livestock health problems (Desjardins and Proctor 2007). In *F. graminearum*, the *TRI* genes are located in three loci: a major 12-gene containing cluster on chromosome 2, a two-gene locus, *TRI1/TRI16* on chromosome 1, and the single gene *TRI101* on chromosome 4 (Alexander *et al.* 2009). A functional *TRI16* is responsible for the production of T-2 toxin in *F. sporotrichioides* (Alexander *et al.* 2009). Polymorphisms in *TRI8*, located in the major cluster contribute to the chemotype differences between the two strains found in Canada (van der Lee 2015; Ward

et al. 2008). Included in the major cluster are the two regulatory genes: *TRI6* and *TRI10*. Both *TRI6* and *TRI10* are necessary for trichothecene biosynthesis, and deletion of either of these two genes renders *F. graminearum* unable to produce DON or its derivatives (Kimura *et al.* 2007; Seong *et al.* 2009; Nasmith *et al.* 2011).

1.1.3.2 Regulation of Secondary Metabolism

The production of secondary metabolites by *F. graminearum* is dependent on the surrounding environment (Sieber *et al.* 2014). Abiotic stressors alter the expression of secondary metabolism genes (Ponts 2015). These stressors include the source of carbon and nitrogen, pH, osmolarity, reactive oxygen species (ROS), and light (Jiao *et al*. 2008; Gardiner *et al.* 2009a; Gardiner *et al.* 2009b; Giese *et al.* 2013; Hegge *et al.* 2015; Gu *et al.* 2014; Ponts *et al*. 2007; Ponts *et al*. 2009; Kim *et al*. 2014).

Non-preferred nitrogen sources, such as putrescine and agmatine, induce *TRI* gene expression, while preferred nitrogen sources such as glutamine, asparagine and ammonia suppress DON production (Gardiner *et al.* 2009b). Similarly, non-preferred carbon sources are closely tied to the production of DON. DON biosynthesis is augmented with the supply of sucrose, compared to the preferred carbon source, glucose (Jiao *et al*. 2008). In addition, the presence of ROS, such as hydrogen peroxide, also results in increased *TRI* gene expression, with *TRI6* and *TRI10* upregulated by 8- and 4-fold times, respectively, and other *TRI* genes such as *TRI5* also increased by up to 20-fold (Ponts *et al*. 2007). During initial stages of infection, the host plant triggers an oxidative burst with the production of ROS, presumably to act as secondary messengers to activate plant defense response genes (Wojtaszek 1997). It is possible that fungi have hijacked this

plant response to activate its virulence genes, which include the expression of secondary metabolism gene pathways such as for the synthesis of DON (Ponts *et al*. 2007; Ponts *et al.* 2009).

RNA-sequencing from cultured cells indicates that many SM gene clusters in *F. graminearum* are upregulated under stress conditions (Seong *et al*. 2009, Nasmith *et al.* 2011). Indeed, *TRI6* expression is observed within 6 hours of nitrogen limited stress *in vitro* (Nasmith *et al.* 2011). Similarly, during infection *in planta*, the fungus may experience similar stress conditions and it is likely that signals originating from plants contribute to upregulate virulence factors such as *TRI6* (Audenaert *et al.* 2014; Ponts 2015). Understanding how environment conditions impact gene expression in *F. graminearum* may provide critical information about the infection process.

1.2 Gene Regulation and Epigenetics

There exists a high degree of control over how an organism utilises resources, specifically in response to changing environmental conditions (Jaenisch and Bird 2003). Both anabolism and catabolism processes have evolved into a multi-level, tightly controlled system and although differences exist between kingdoms, eukaryotes regulate gene expression in similar ways (Holstege *et al.* 1998). In an eukaryotic cell, transcription, translation and post-translational control mechanisms together represent levels of regulation that result in many different outputs or phenotypes (Levine and Tjian 2003; Sonenberg and Hinnebusch 2009). The ability to influence a phenotype without an underlying change in genotype is the realm of epigenetics (Handy *et al*. 2011). First coined in 1942 by Conrad Waddington, epigenetics is defined as a study of changes in

gene function that can be mitotically or meiotically inherited but which cannot be explained by changes in the DNA sequence itself (Goldberg *et al.* 2007). The most recent definition, provided by Berger *et al.* (2009) defines an epigenetic trait as one that arises from a change in a gene without alteration in the DNA sequence. The term itself is derived from the Greek prefix, "epi" indicating "on top of" or "over", suggesting a level of control above the gene/transcription level – changes in phenotype without an underlying change in genotype. This section will describe the key pathways used by cells to regulate gene expression by epigenetic mechanisms.

1.2.1 Types of Epigenetic Regulation

Phenotypes that arise through epigenetic changes are likely triggered by environmental conditions (Berger *et al.* 2009). Environmental changes can be considered the epigenetic originator and the signal transduced by the 'epigenetor' activates signalling pathways that include protein-protein interactions and other protein modifications (Berger *et al.* 2009). These signals are transient but exist long enough to trigger epigenetic changes in a cell (Stankiewicz *et al*. 2013). Changes are usually manifested as alterations to the so-called 'epigenome', represented by the higher order DNA structure and packaging into chromatin (Stankiewicz *et al.* 2013). Alterations in the epigenome can change the accessibility of the DNA and give rise to the epigenetic phenotype (Carlberg and Molnar 2016). DNA binding elements such as transcription factors and non-coding RNAs (ncRNA) are examples of epigenetic initiators (Piferrer 2013; Carlberg and Molnar 2016). As an example, ncRNA Xist is involved in the silencing of the mammalian X chromosome (Berger *et al.* 2009; Sharp *et al.* 2011). This results in transcriptional

silencing of one of the female X-chromosomes, and subsequently dose equivalency to males (Morey and Avner 2010).

The initiator signal may be transient, but changes brought on by the initiator may also persist through the epigenetic "maintainer", which sustains the chromatin state through the cell cycle or even throughout the organism's lifecycle (Berger *et al.* 2009). These epigenetic mechanisms provide evidence that mutation in the nucleotide sequences are not the only heritable form of genetic information contained in the cell (Martienssen and Colot 2001).

1.2.1.1 "Initiators" of Epigenetics

Diverse classes of RNA exist in eukaryotes, ancillary to the better known messenger RNAs (mRNAs; Morris and Mattick 2014). These include non-coding RNA, typically subdivided into two classes: small ncRNA (sncRNA) are usually 21-25 nucleotides long, but can range up to 300 nucleotides in size, and long ncRNA (lncRNA) are >300 nucleotides and up to thousands of nucleotides in length (Costa 2010; Cheng *et al.* 2012; Holoch and Moazed 2015). Small ncRNAs are composed micro RNA (miRNA), small interfering RNA (siRNA), piwi-interacting RNA (piRNA) and small nucleolar RNA (snoRNA) (Esteller 2011). They are involved in myriad functions including in translational silencing, suppression of repetitive elements and modification of other RNA molecules. miRNA is an example of regulation by ncRNA. The primary mechanism for miRNA is to bind to the 3' untranslated region (UTR) of protein coding genes, resulting in either translational repression or mRNA degradation (Ling *et al.* 2013). Other less common mechanisms include binding to the 5' UTR, which may either

result in translational repression or activation (Ling *et al.* 2013). Both mechanisms may result in cellular phenotype changes, with no changes in the genotype. Recent studies have implicated ncRNAs such as miRNA as a possible driver of evolution in brain development of human and other primates through post-transcriptional gene silencing (PTGS) mechanisms, suggesting the importance of epigenetics in evolution (Berezikov *et al*. 2006).

Non-coding RNAs have also emerged as important "initiators" in regulation of chromatin structure in eukaryotes (Holoch and Moazed 2015). One example is piRNA, a member of sncRNA class that is transcribed from genome regions containing transposable or other repetitive elements (Esteller 2011). piRNAs interact with PIWI proteins to suppress transposable elements (TEs) through two mechanisms: cleavage of TEs by PIWI proteins, or through chromatin reorganization by heterochromatin-mediated gene silencing and DNA methylation (Esteller 2011). In addition to the previously mentioned long ncRNA *XIST* that is involved in X-chromosome silencing, other lncRNAs function in the regulation of the telomeres and in genetic imprinting (Esteller 2011). These lncRNA are able to recruit chromatin remodeling complexes to specific loci, for example the *Hox* transcript antisense RNA (*HOTAIR*) originates in the *HOXC* locus in humans and silences transcription in 40kb of the *HOXD* locus by inducing a repressive chromatin state (Mercer *et al*. 2009). Also, disruption and dysregulation of ncRNAs have been implicated in tumorigenesis and other neurological, cardiovascular and developmental diseases in humans (Esteller 2011).

Epigenetic initiators can also include DNA binding proteins (Araki and Mimura 2017). In mammals, five methylcytosine (5mC) binding proteins (MBDs) have been

identified: MBD1-4 and MeCP2 (Saito and Ishikawa 2002). The MBD proteins recruit chromatin modifying complexes and deposit epigenetic marks that initiate the epigenetic signal (Fujita *et al.* 1999; Saito and Ishikawa 2002).

Epigenetic drivers such as ncRNA or DNA binding proteins are clearly important, yet their role in the generation of the epi-phenotype is poorly understood. ncRNA appears to function primarily as epigenetic initiators and may interact with epigenetic maintainers to propagate the epigenetic signal over time. DNA binding proteins may recruit nucleosome remodeling complexes, or produce changes to epigenetic marks on histone proteins, thus altering chromatin structure (Fujita *et al.* 1999; Saito and Ishikawa 2002).

1.2.1.2. "Maintainers" of epigenetics

Epigenetic maintainers are comprised of chromatin/nucleosome remodeling complexes, histone modifications and DNA methylation (Araki and Mimura 2017). Eukaryotic genetic information is tightly packaged into the nucleus and arranged into chromosomes. Chromosomes are composed of repeating units of nucleosomes, which in turn are made up of ~146bp of DNA wrapped around 8 histone proteins, the histone octamer (Elgin and Weintraub 1975; Zlantanova and Thakar 2007). In this way, chromatin is a combination of protein and DNA that together package to maintain chromosomal DNA in a higher order structure. Chromatin can typically be subdivided into heterochromatin and euchromatin: heterochromatin is composed of tightly packed, condensed nucleosomes, while euchromatin maintains a looser, more open structure (Murakami 2013). Euchromatin domains typically define more transcriptionally active and accessible areas of the genome, while heterochromatin domains are in contrast

generally inaccessible and transcriptionally silent (Grewal and Moazed 2003). In many eukaryotes, heterochromatin can be further subdivided into facultative and constitutive heterochromatin. Constitutive heterochromatin is stably maintained and is typically localised to repeated sequences and transposons, whereas facultative heterochromatin is most commonly found in repressing protein coding genes, in addition to being regulated by the environment and through development (Murakami 2013). Thus, organisation of genetic information into chromatin structure avails itself to epigenetic regulation, thereby influencing gene expression (Grewal and Moazed 2003; Becker and Workman 2013). As epigenetic initiators modify the chromatin structure, epigenetic maintainers preserve and perpetuate these changes through life cycles and generations (Berger *et al.* 2009).

Nucleosome remodeling complexes are multifunctional ATP-dependent protein complexes that introduce a level of plasticity into how nucleosomes package DNA. These complexes perform a variety of functions and can alter accessibility through nucleosome translocation, nucleosome removal or histone exchange (Eberharter and Becker 2004). Remodeling complexes such as the ISWI (Imitation Switch) family exhibit nucleosome translocation activity, resulting in the transfer of a histone octamer from one nucleosome region to another previously "naked" DNA region (Lorch 1999; Fazzio and Tsukiyama 2003). Nucleosomes can also be removed from the chromatin structure. Nucleosome remodeling complexes such as RSC (Chromatin Structure Remodelling) are able to remove promoter nucleosomes, in addition to performing nucleosome translocation (Lorch *et al.* 2011). Histone exchange is also a dynamic method for altering nucleosome positioning. The histone variant H2AZ is a variant of histone H2A, sharing only 60% sequence identity while being highly conserved across species from protozoans to higher

eukaryotes (Zlantanova and Thakar 2007). The chromatin remodeling complex SWR1 (Switch/Sucrose non fermentable-related) in *Saccharomyces cerevisiae* is capable of exchanging the conventional histone H2A with the H2AZ variant, resulting in changes to the chromatin state (Mizuguchi *et al.* 2004). Mutant strains of *htz1* (a component of SWR1 complex) result in differential regulation of several hundred genes, suggesting that histone substitution is a key regulator of chromatin structure and DNA accessibility (Meneghini *et al.* 2003). Additionally, substitution of histone variants has been shown to prevent the spread of facultative heterochromatic regions in the genome, suggesting this mechanism may also play a vital role in gene regulation. Interestingly, studies suggest that H2AZ variant functions antagonistically with another epigenetic regulator in *Arabidopsis thaliana* - DNA methylation (Zilberman *et al.* 2008).

As building blocks of the nucleosomes, two copies of each histone protein, H2A, H2B, H3 and H4 compose the basic histone octamer, allowing histones to play a crucial role in establishing hetero- or eu-chromatin structure (Grewal and Moazed 2003). Post-translational modifications (PTMs) of the core histones can fundamentally alter the organisation of DNA into chromatin, thereby implicating these modifications in the regulation of many DNA processes including DNA repair, transcription, and replication (Tessarz and Kouzarides 2014). Typically modifications occur on the N-terminal histone tails. These tails are not essential for protein stability, but have been identified to make secondary and flexible contacts with surrounding nucleosomes and DNA (Cheung *et al.* 2000). Additionally, since these regions protrude from the nucleosome, they are easily accessible for interaction with signalling molecules (Roth *et al.* 2001). In this way, the N-terminal tails of histones with different PTMs can potentially interact with multiple

signalling components and give rise to the myriad downstream responses (Cheung *et al.* 2000).

The covalent modifications on histones are comprised of at least eight different classes, including acetylation and methylation that may occupy over 60 different residues on the N-terminal tails (Jenuwein and Allis 2001; Kouzarides 2007). Some residues are able to be modified multiple times; the lysine residue can be found as either mono-, di- or tri-methylated, while the arginine residue has been observed to be either mono- or di-methylated (Bannister and Kouzarides 2011). The massive array of possible modification types and locations suggests a wealth of epigenetic information that is contained within histone modifications. These modifications, monitored through chromatin immunoprecipitation (ChIP or ChIPseq) operate by two main (known) mechanisms: alteration of chromatin contacts or recruitment of non-histone enzyme complexes to the chromatin (Kouzarides 2007; Schwammle *et al.* 2014). The alteration of chromatin contacts is believed to be caused primarily through charge changes on the N-terminal tails (Kouzarides 2007). Acetylation of lysine residues can neutralise positive charge, weakening the binding between the histone and the negatively charged DNA (Dong and Weng 2013). These modifications can have a turnover rate of minutes after a stimulus reaches the cell surface (Kouzarides 2007). The speed and variety of histone modifications suggest a strong role in response to environmental signals and translating it into changes in chromatin structure.

The recruitment of nucleosome remodeling protein complexes is dependent on the type of histone modifications. Histone methylation is recognised by chromo-like domains (~60 aa), while histone acetylation is recognised by bromodomains (~110 aa), and histone

phosphorylation is recognised by a domain within 14-3-3-like proteins (Jenuwein and Allis 2001; Kouzarides 2007). Although exceptions exist, histone acetylation is associated with euchromatin and active expression, and conversely, histone methylation with heterochromatin and gene silencing (Kouzarides 2007; Graff and Tsai 2013). Histone phosphorylation is implicated in the formation of both euchromatin and heterochromatin structures, depending both on the placement of the epigenetic mark and interaction with any surrounding modification marks (Rossetto *et al.* 2012). The incredible number and variety of histone modifications and modification sites make histones a key target in studying epigenetic effects on gene expression.

While both nucleosome remodeling and histone modifications have both been implicated in higher order chromatin structures that alter the accessibility of DNA, another epigenetic mark, DNA methylation, may also play an important role. DNA modifications, usually N-6 methyladenine (6mA), and C-5 methylcytosine (5mC), are the most common DNA epigenetic marks found in higher eukaryotes (Pfeifer 2016). In prokaryotes, 6mA is the most prevalent DNA modification, involved in the host defence system (Luo *et al*. 2015). Due to the abundance and widespread nature of 5mC modifications in mammals and plants, only recently has 6mA been identified in eukaryotes and much remains unknown about this epigenetic mark (Luo *et al*. 2015). For example, both *Caenorhabditis elegans* and *Drosophila melanogaster* harbour 6mA, suggesting a role for 6mA in eukaryotic species, however, its function remains largely unknown (Luo *et al*. 2015). A leading hypothesis stems from the observation that 6mA destabilises Watson-Crick base pairing in RNA, which alters the RNA structure and affects protein binding (Luo *et al*. 2015). A similar mechanism could exist for 6mA in

DNA, which in turn may impact transcription or replication. Both 6mA and 5mC may also facilitate or inhibit recognition of DNA-binding proteins such as transcription factors, thereby causing changes in gene expression (Jaenisch and Bird 2003; Luo *et al*. 2015).

Unlike 6mA epigenetics, 5mC has been intensively studied in mammals as aberrant 5mC modifications have been identified in a wide variety of human diseases, including cancer (Robertson 2005). Methyl-cytosine is involved in regulating a number of important cellular processes, including transcription, chromatin structure, X chromosome inactivation, genomic imprinting and chromosome stability (Robertson 2005). It should be noted that DNA methylation is not an essential mark in all eukaryotes, for example *C. elegans* is void of 5mC methylation (Schubeler 2015).

In mammals, DNA methylation occurs after replication and the extent of methylation changes through development (Jaenisch and Bird 2003). DNA demethylation is extensive in mammals during early development and cleavage (2-3 days after fertilisation), followed by genome-wide *de novo* methylation after implantation (8-9 days after fertilisation). In the male genome, DNA methylation is stripped just hours after fertilisation (Jaenisch and Bird 2003). After gastrulation, *de novo* methylation is limited, except in conditions of disease, such as cancer (Jaenisch and Bird 2003). DNA modification to 5mC occurs in three genomic contexts, CG, CHG and CHH, with 'H' representing cytosine, adenine or thymine. CG methylation is the most common context for higher eukaryotes, however, all three contexts are observed in plants (Krueger and Andrews 2011; Gehring and Henikoff 2007). How these different contexts influence DNA methylation or gene expression is still poorly understood. DNA methylation in

symmetrical contexts, such as CG or CHG can be propagated through DNA replication, with maintenance methyltransferases adding 5mC to the daughter strand (Finnegan *et al.* 2000). There is currently no known mechanism for the propagation of asymmetrical 5mC (CHH), as these are methylated *de novo* on the daughter strand (Finnegan *et al.* 2000). In higher eukaryotes, methylation patterns are also tissue specific with the majority of methylation occurring in the CG context, accounting for 70-80% methylation in somatic tissues (Li and Zhang 2014). In plants, methylation content varies widely, with plants such as *Arabidopsis* methylated at ~32.4% of total cytosines, and wheat germ methylated at >20% of total cytosines (Cokus *et al*. 2008). This variability is largely due to abundances of repetitive elements in the genome, with repetitive elements being typically hyper-methylated (Gehring and Henikoff 2007). The majority of 5mC in *Arabidopsis* for actively transcribed genes occurs within the gene coding region. Conversely, methylation of the 5' region/promoter region correlates with genes that are expressed at lower levels, similar to what is observed in mammals (Gehring and Henikoff 2007; Smith and Meissner 2013).

In fungi, modification of DNA occurs on both cytosine (5mC) and adenine (6mA) bases (Seidl 2017; Mondo *et al.* 2017). The 5mC methylation in eukaryotes tends to function as a repressive epigenetic mark, limiting transcription (Schubeler 2015). Interestingly, 6mA methylation marks in early-divergent fungi such as *Hesseltinella vesiculosa* are prevalent in areas of active genes, a role contrasting the function of 5mC methylation (Mondo *et al*. 2017). Some fungal species, including the Dikarya (Ascomycota and Basidomycota), show increased prevalence of 5mC modifications, however, dramatic differences in methylation levels have been reported among fungi.

Saccharomyces cerevisiae and *Schizosaccharomyces pombe* lack 5mC, whereas *Aspergillus flavus* is extremely limited or absent in 5mC. In contrast, up to 4% were identified as 5mC in *Armillaria bulbosa* (Liu *et al.* 2012; Capuano *et al.* 2014; Binz *et al*. 1998). To date, the most extensively studied fungus for DNA modification is the genome of *Neurospora crassa*, where ~1.5-3% of cytosines are methylated (Foss *et al.*1993; Tamaru and Selker 2001; Selker *et al*. 2002; Aramayo and Selker 2013; Seymour *et al*. 2016).

DNA methylation is the product of DNA methyltransferases (DMTs). These enzymes, coupled with methyl donor S-adenosyl-methionine, are responsible for the deposition of DNA methylation on cytosine residues (Jin and Robertson 2013; Zhong *et al.* 2014; **Figure 2**). In brief, methylation begins with the nucleophilic attack of the C-6 position on the cytosine base by the DNMT. Electrophilic addition of the methyl-group followed by de-protonation of C-5 carbon then results in the generation of the methylated cytosine base.

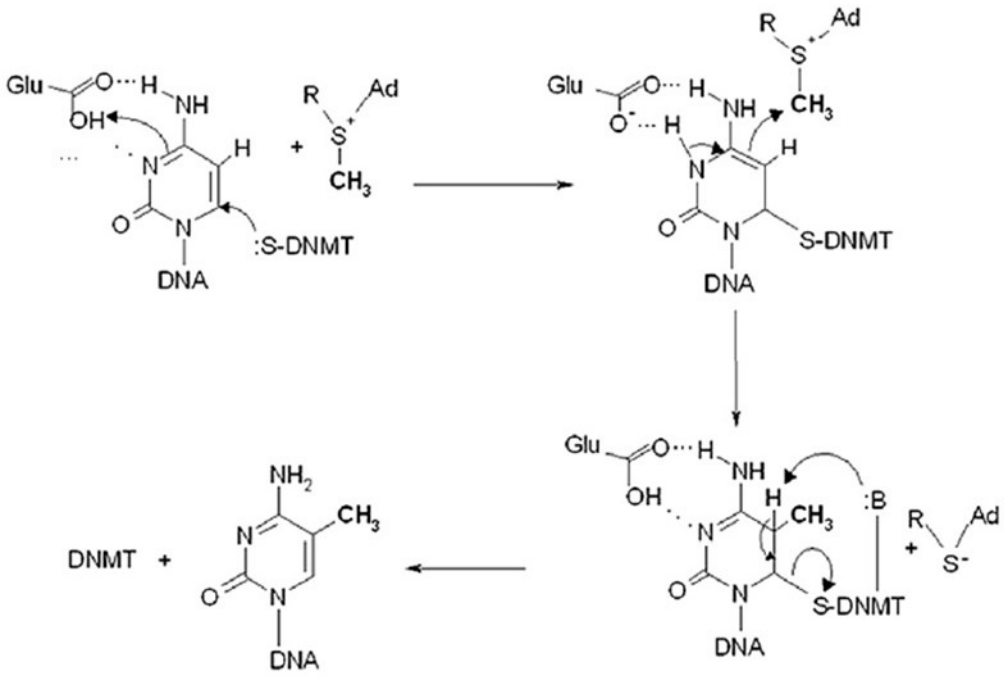


Figure 2: Mechanism of DNA methylation deposition by DNA methyltransferase enzymes in conjunction with the methyl-donor, *S*-adenosyl-methionine. Figure adapted from Plitta *et al.* (2012).

Many higher eukaryotes, including plants and mammals, contain a number of DNA methyltransferase families, including: DNMT1, DNMT2, DNMT3, and DNMT4, following the nomenclature for human DMTs (Ponger and Li, 2015). The DNMT1 family encode for enzymes that maintain the epigenetic signal throughout the cell cycle and DNA replication. Many of these enzymes contain a bromo-adjacent homology domain, which allows interaction with DNA (Ponger and Li, 2015). A recent study indicated that DNMT1 family methyltransferases may be capable of *de novo* methylation activity, in addition to its maintenance activity (Jeltsch and Jurkowska 2014). Fungal DMTs, such as*Nc*DIM-2 (Deficient in methylation) of *Neurospora crassa* are typically grouped with the DNMT1 family (Ponger and Li, 2015). The DNMT2 family, present in chordates and plants, has weak or no DNA methylation activity (Ponger and Li, 2015). The DNMT3 family is also present in chordates and plants and show the highest levels of *de novo* methylation (Ponger and Li, 2015). The DNMT4 family is poorly understood, but is typically comprised of the fungal DNA methyltransferases such as the DmtA and RID (Repeat induced deficient). This family has not been characterized for either *de novo* methyltransferase or its maintenance activities (Ponger and Li, 2015; Yang *et al.* 2016).

By sequence similarity, two main families of DMTs have been identified in fungi: DNMT4 and DNMT1 (Yang *et al.* 2016). The DNMT1 family includes maintenance methyltransferases from plant and animal species and these methyltransferases are tasked to maintain the epigenetic signal through DNA replication by deposition of methylation marks on daughter strands from symmetrical (CG, CHG) cytosines in the parent strand (Ponger and Li, 2015). Among the DMTs identified in eukaryotic species, fungal DMT1s

appear to be the most divergent (Kouzminova and Selker 200; Ponger and Li 2015). An example of divergence is exemplified by *Nc*DIM-2. Although the length of both the N and C-terminal regions is most similar to DNMT1, the *Nc*DIM-2 shows poor sequence homology at the N-terminus to other DNMT1 class DMTs (Kouzminova and Selker 2001). *Nc*DIM-2 also lacks the dipeptide repeat that links the N- and C-terminal domains found in all other DNMT1s (Kouzminova and Selker 2001). Studies suggest that the *Nc*DIM-2 is responsible for both maintenance and *de novo* methylation, atypical of the DNMT1 class of DMTs (Kouzminova and Selker 2001; Selker *et al.* 2002). As mentioned previously, methylation maintenance depends on the symmetrical nature of methylated sites (CG and CHG) while methylation at non-symmetrical sites (CHH) is a product of *de novo* DNA methylation (Selker and Stevens 1985; Selker *et al.* 1987b). DNA methylation in *N. crassa* is prevalently methylated at non-symmetrical CHH sites, further suggesting the role of a *de novo* methyltransferase activity of *Nc*DIM2 (Kouzminova and Selker 2001).

The DNMT4 family include *Af*DmtA from *Aspergillus fumigatus*, *Ai*Masc1 from *Ascobolus immersus* and *Nc*RID from *N. crassa* (Freitag *et al.* 2002; Yang *et al.* 2016). In *N. crassa*, DNMT4 is well characterized as a repeat-induced (RIP) DMT, although its methyltransferase capacity is unknown (Freitag *et al.* 2002). The RIP mechanism in fungi is used to hyper-mutate repetitive sequences and prevent the movement and spread of transposable elements in the genome by generating C: G to A: T transition mutations (Freitag *et al.* 2002). *Nc*DMT4 is crucial to the RIP mechanism by means of unrepaired deamination of potentially methylated cytosine to uracil or thymine (Freitag *et al.* 2002). Alternatively, DNMT4 family DMTs methylate DNA after it has been RIP'd (Lewis *et al.*

2009). *Ai*Masc1 in *A. immerses* is predicted to be involved in the silencing of duplicated genes during the fungal sexual cycle, a process known as 'methylation induced premeiotically' (MIP). This process involves *de novo* methylation of cytosines prior to the sexual cycle (Chernov *et al.* 1997). Homologues of both DNMT1 and DNMT4 are found in the rice pathogen, *Magnaporthe oryzae*, suggesting conservation between fungal species (Jeon *et al.* 2015). The fungal DMTs will be covered in more detail in upcoming chapters.

1.2.2 Role of de-methylation in epigenetics

DNA methylation represents a stable and heritable epigenetic mark. However, the organization of the genome faces the competing demands of stability and flexibility (Kohli and Zhang 2013). DNA de-methylation allows for adaptive control over this epigenetic process, resulting in a balance between stability and flexibility (Kohli and Zhang 2013).

De-methylation can occur through both passive and active mechanisms (Piccolo and Fisher 2014). Passive mechanisms of DNA de-methylation refer to the loss of methylation upon successive DNA replication in the absence of DNA maintenance machinery (Kohli and Zhang 2013). Passive de-methylation involves the progressive dilution of DNA methylation through DNA replication; exclusion of DMTs from the nucleus during replication prevents the propagation of DNA methylation (Law and Jacobsen 2010). Passive de-methylation is observed during early development in humans, at which time the primary maintenance methyltransferase (DNMT1) is retained in the cytoplasm and prevented from binding to DNA in the nucleus (Cardoso and Leonhardt

1999). Active de-methylation occurs by three hypothetical routes: the methyl group can either be removed, or the cytosine is excised and repaired, or the 5mC can be chemically modified (Piccolo and Fisher 2014). A common example of active de-methylation in plants and animals involves the oxidation of mC to hydroxyl-methylcytosine (5hmC) by TET (Ten-eleven translocation) enzymes, followed by a series of chemical reactions leading to 5-formylcytosine (5fC) or 5-carboxylcytosine (5caC), both of which can be converted into an unmodified cytosine by TdT (Terminal deoxynucleotidyl transferase) enzymes (Ito *et al.* 2010; Ito *et al*. 2011). Another process for active de-methylation involves 5mC removal by 5mC specific glycosylases in combination with base excision repair (BER) mechanisms (Law and Jacobsen 2010).

1.3 Project Outline

DNA methylation is a widespread epigenetic mark regulating many important processes such as transcription, chromatin structure and chromosome stability. As such, we hypothesized that DNA methylation may be integral to many aspects of the fungus, including production of SMs, fungal development and pathogenicity. The overall objective of this thesis was to determine a possible role for DNA methylation in both fungal development and pathogenicity in *Fusarium graminearum*. Chapter 2 investigated the role of the two DMTs: FGSG_10766 (DNMT1) and FGSG_08648 (DNMT4), in the fungal pathogen *F. graminearum*. Specifically, we investigated their role in SM regulation/production, developmental processes such as sexual and vegetative growth, and in virulence of this pathogen. Chapter 3 of this thesis examined the presence and pattern of the fungal methylome by whole genome bisulfite sequencing (WGBS). WGBS

will be used to assess the role of the environment in establishing DNA 5mC methylation marks across three genomic cytosine contexts, CG, CHG and CHH.

Chapter 2: DNA Methyltransferases: Gardeners of the Epigenome

Chapter 2 – Introduction

2.1 Fungal DNA Methyltransferases

DNA methylation is a common epigenetic mark found in diverse organisms ranging from bacteria to plants, mammals and fungi (Kouzminova and Selker 2001; Zhang *et al*. 2006; Eckhardt *et al.* 2006; Romero *et al*. 2015). Methylation of cytosine bases (5mC) can occur in 3 contexts: CG, CHG and CHH (H= A, T, C), with CG methylation being the most common context in mammals, and all three contexts common in plants (Krueger and Andrews 2011; Gehring and Henikoff 2007). The deposition of methylation via DNMT1 occurs on cytosine in all three sequence contexts (CG, CHG and CHH;Kouzminova and Selker 2001; Chan *et al.* 2005; Law and Jacobsen 2010). DNA methylation is the product of DNA methyltransferases (DMTs) in association with the methyl-group donor *S*-adenosyl-methionine (Jin and Robertson 2013; Zhong *et al.* 2014). In brief, the methylation of the cytosine base occurs following electrophilic attack on C-6 by the DMT active site methionine residue, generating a reactive covalent adduct. This adduct can readily undergo electrophilic addition of the methyl-group from the donor, *S*-adenosyl-methionine. De-protonation of the C-5 position restores the double bond and removes the DMT enzyme (**Figure 2**).

DMTs are grouped by domain architecture in addition to sequence similarity (Ponger and Li 2015). Fungal DMTs primarily fall into two families of DNA methyltransferases, DNMT1 and DNMT4 (Ponger and Li 2015). DNMT1s in fungi include DIM-2 (e.g. *N. crassa*, *Neurospora tetrasperma*, *M. oryzae*) and is required for

DNA methylation (Kouzminova and Selker 2001). Fungal DNMT1 have been shown to possess limited *de novo* DNA methyltransferase activity (Kouzminova and Selker 2001; Fatemi *et al.* 2002; Jeltsch and Jerkowska 2014). The DNMT1 family members in higher eukaryotes, including plants and mammals, contain a number of conserved domains, including a C-5 cytosine methyltransferase domain, a DMAP1 (DNMT1 Associate Protein) -binding domain, a C-5 cytosine methyltransferase 1 replication foci domain, a Zinc finger (CXXC-type) domain, and a bromo-adjacent homology domain (Yang *et al.* 2016). The domain architecture of a DMT provides valuable information toward understanding its function. For example, the DMAP1 binding domain allows DNMT1 to interact with DMAP1 and HDAC2 (Histone Deacetylase), which act together as a transcription repressor complex (Rountree *et al.* 2000). The replication foci domain and zinc finger domain allow the maintenance of methylation, establishment of heterochromatin, and de-acetylation of histone proteins at newly replicated DNA (Rountree *et al.* 2000). Studies have identified that DMAP1 enhances DNA methylation deposition (Lee *et al.* 2010). Fungal DNMT1, such as Dim-2 in *M. robertsii* or *N. tetrasperma,* diverge from other eukaryotic DNMT1s as they lack the conserved domains except for the bromo-adjacent homology domain and the main C-5 cytosine methyltransferase domain (Yang *et al.* 2016).

The DNMT4 family is exclusively found in fungi, including DmtA from *Aspergillus* spp., RID in *N. crassa* and Masc1 in *Ascobolus immerses* (Chernov *et al.* 1997; Freitag *et al.* 2002; Yang *et al.* 2016). This group of DMTs contain only a single C-5 DNA methyltransferase domain (Yang *et al.* 2016). Although DNMT4 has the domain required for DNA methylation, to date there is little evidence to show that these DMTs

actively methylate DNA *de novo* or have maintenance capacity (Lee *et al.* 2008; Yang *et al.*2016). Disruption of RID in *N. crassa* resulted in defects in the repeat induced point mutation (RIP) process, but no changes in methylation patterns were detected (Freitag *et al.* 2002). Similarly, no methylation has been ascribed to DmtA in *Aspergillus flavus* (Liu *et al*. 2012). Overall, evidence suggests that DNMT4 plays only a small role in DNA methylation, if any, and may play other more important roles. For example, deletion of *dmtA* in *A. flavus* results in a wide variety of phenotypes including conidiation and aflatoxin biosynthesis deficiencies (Yang *et al.* 2016). DNMT4 functions in silencing of repeat sequences (RIP) in *N. crassa* and silencing of duplicated genes (methylation induced premeiotically, MIP) in *A. immerses* (Freitag *et al.* 2002; Chernov *et al*. 1997). The RIP process functions in hyper-mutation of repetitive sequences by means of unrepaired deamination of C or methyl-C (5mC) to T (Freitag *et al*. 2002). Two mechanisms for DNMT4 have been proposed in RIP. The first mechanism involves the *de novo* methylation of C to 5mC, which is thought to be 10^4 times more likely to spontaneously deaminate into T than unmodified C (Selker 1990). The second proposed mechanism suggests that DNA is methylated by DNMT4 after repeat induced point mutation because A:T rich RIP'd DNA is more efficient to direct methylation of histone 3, lysine 9 (H3K9), a key player in deposition of DNA methylation (Lewis *et al.* 2009). It is possible that these enzymes function almost exclusively in methylation of C bases that are quickly deaminated into T, and as such are rarely observed. The MIP process, however, is involved in hyper-methylation and silencing of sequence duplications without mutation (Wolffe and Matzke 1999). This indicates that DNA methylation by DNMT4 occurs independently of mutation.

The mechanism for DNA methylation in a maintenance capacity is well documented in fungi (Freitag *et al.* 2004; Honda *et al.* 2012; Du *et al.* 2015; Tamaru *et al.* 2003; Lewis *et al.* 2010b). In brief, histone methyltransferase DIM-5 (deficient in methylation - upon deletion of this enzyme), in association with the DCDC complex (Dim-5/-7/-9/CUL4/DDB1) is responsible for the deposition of tri-methylation at lysine 9, histone 3 (H3K9; Tamaru *et al.* 2003; Lewis *et al.* 2010b). Heterochromatin protein 1, a chromo-domain protein, recognizes the tri-methylation signature and acts as an adaptor to other DMTs (Freitag *et al.* 2004; Du *et al.* 2015). In the presence of the methyl group donor, *S*-adenosyl methionine, this interaction results in the methylation of the surrounding DNA (Jin and Robertson 2013; Zhong *et al.* 2014).

Epigenetics in fungi has been under investigation primarily in the model organism *N. crassa* (Selker *et al*. 1987; Selker 1990; Kouzminova and Selker 2001; Freitag *et al*. 2002). The study of epigenetics in plant pathogenic fungi, however, has lagged behind. A study in *A. flavus* indicated that its genome lacked DNA methylation (Liu *et al.* 2012). However, a recent study in the same fungus revealed decreased conidiation, sclerotial production, aflatoxin biosynthesis and virulence upon deletion of *dmtA* (*AfDNMT4*) (Yang *et al.* 2016). This suggests additional research is necessary to understand the relationship between DNA methylation and pathogenesis. In the rice pathogen, *M. oryzae*, DNA methylation was shown to be dispensable for pathogenicity (Jeon *et al*. 2015). These studies have conflicting views of DNA methylation in plant pathogenic fungi, and as such further research into the role of epigenetics in fungi is necessary.

In *F. graminearum*, histone epigenetics plays a significant role in regulating both secondary metabolism and pathogenicity (Connolly *et al.* 2013). The study showed that

the removal of methylation mark of histone 3 resulted in activation of 14% of genes in the genome, including genes involved in SM. This underscores the importance of studying epigenetics in plant pathogenic fungi, like *F. graminearum,* and to date no study has shown a link between DNA methylation and its effect on either SM or pathogenicity in *F. graminearum*. This study examines the role of DNA methyltransferases *Fg*DNMT1 and *Fg*DNMT4 in development and pathogenicity of *F. graminearum.*

Chapter 2 - Methodology

The *F. graminearum* strain DAOM233423 was used as wild-type (*WT*) in all experiments. Experiments were initiated using fresh macroconidia generated in carboxymethylcellulose (CMC) medium (Walkowiak *et al.* 2015). One individual transformation strain was tested for each gene deletion, as complementation work is on-going.

2.2.1 DNA Methyltransferases in *F. graminearum*

DNA methyltransferases DNMT1 and DNMT4 in *F. graminearum* were identified through the manually curated genome database MIPS (Munich information center for protein sequences) at the German Research Center for Environmental Health (pendant.gsf.de), as well as reciprocal BLAST (basic local alignment search tool) searches to closely related fungal species such as *N. crassa* and the rice pathogen *M. oryzae*. No additional cytosine specific DNA methyltransferases were identified by either method. Domain searches of DNMT1 and DNMT4 were performed using InterPro (https://www.ebi.ac.uk/interpro/) to identify the presence of DNA methyltransferase domains.

2.2.2 Phylogenetic Analysis of DNA Methyltransferases

Protein sequences for 35 eukaryotic DNA methyltransferases were identified from Yang *et al.* (2016) and obtained from NCBI (https://www.ncbi.nlm.nih.gov/protein/). Phylogenetic relationships were inferred using the Neighbor-Joining method to generate

an appropriate starter tree (Saitou and Nei 1987). The Maximum-Likelihood method (ML) was then used under the WAG model for protein substitution and default settings in CLC-Genomics Workbench (Qiagen Bioinformatics v9.5.3) to create the final tree (Whelan and Goldman 2001). Each clade was supported by bootstrap consensus inferred from 1000 replicates (Felsenstein 1985).

2.2.1 Generation of *F. graminearum* Transgenic Strains

Mutant strains of DNA methyltransferase genes *DNMT1* and *DNMT4* were generated by the USER (Uracil-Specific Excision Reagent) friendly cloning system in conjunction with *Agrobacterium tumefaciens* (LBA4404), as outlined in Frandsen *et al*. (2008). A brief summary of this protocol can be seen in **Supplemental Figure F1**. In short, PCR amplification of ~1 kb gene-flanking regions with USER specific primers (**Supplemental Table T1**) were cloned into either pRF-HU2 vector with hygromycin resistance or GU2 vector with geneticin G418 resistance (Frandsen *et al.* 2008). Homologous recombination of the flanking regions in the fungi was facilitated by transforming fresh *F. graminearum* spores with *A. tumefaciens*. The transformation results in the replacement of the target gene by either hygromycin or geneticin resistance markers. A double deletion strain, Δ*dnmt1*/Δ*dnmt4* was created in similar fashion, using Δ*dnmt1* as the background strain. A Δ*dnmt4:DNMT4* was constructed to complement the deleted *dnmt4* strain with the pRF-GU vector.

2.2.1.1 RNA Extraction and PCR Analysis

Total fungal RNA from 24 hours nutrient rich (NR) growth was extracted from mycelia using TRIzol Reagent (Invitrogen), purified using the InviTrap Spin Cell RNA Mini Kit (Stratec, Germany), and then converted into cDNA (1 μg) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). RT-qPCR reactions were performed in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems) and the QuantStudio3 qPCR machine (Applied Biosystems) according to the manufacturer's instructions. A list of qPCR primers can be found in **Supplemental Table T1**.

All deletion strains were screened by PCR to confirm the absence of the target genes and the presence of the resistance marker. Touch-down PCR conditions: 100 ηg of genomic DNA for 8 cycles (63˚C to 55˚C) and for 22 cycles at 55˚C. qPCR was used to assess copy number of geneticin in Δ*dnmt1* and Δ*dnmt1*/Δ*dnmt4* strains. Standard curves were constructed for geneticin and β-tubulin primer sets using a ten-fold dilution series ranging from 15 to 0.015ηg from genomic DNA of Δ*tri6*/Δ*mcc*, containing one copy of geneticin and one copy of *β*-tubulin (*FGSG_09530*; Subramaniam *et al*. 2015). For quantification, 1 ηg of genomic DNA was used from each strain. Δ*tri6*/Δ*mcc* served as internal control and β-tubulin as the internal reference, in accordance with previous research (**Supplemental Figure F1**). All samples were assessed in triplicate using the QuantStudio3 (Applied Biosystems). Primers can be found in **Supplemental Table T1**. Transcript levels of *DNMT1* in Δ*dnmt1* and Δ*dnmt1*/Δ*dnmt4* were also assessed using RT-qPCR (**Supplemental Figure F1**).

Successful transformants were selected as a single spore, reconfirmed, and submitted to the Canadian Collection of Fungal Cultures.

2.2.2 Phenotypic Analysis of Transgenic *F. graminearum*

A number of assays were employed to determine the role of *DNMT1* and *DNMT4*. We used both single and double mutants of Δ*dnmt1,* Δ*dnmt4* and Δ*dnmt1*/Δ*dnmt4*, as well as the complemented strain Δ*dnmt4:DNMT4* to asses various developmental phenotypes such as vegetative and sexual development in comparison to the *WT*. The mutant strains were also used to profile SM production and used in pathology tests to assess virulence on wheat.

2.2.2.1 DON Analysis & Quantification

The mutant strains were assessed for the ability to produce 15-ADON compared to the wild type strain. This protocol is adapted from Miller and Blackwell (1986). An inoculum of 5,000 spores mL^{-1} of each strain were inoculated into VWR 6 well Tissue Culture Plates containing 4 mL of NR growth media (NR – 56mM NH_4Cl, 8.1 mM $MgSO_4$ $7H_2O$, 0.23 mM $FeSO_4 \cdot 7H_2O$, 14.7 mM KH_2PO_4, 2 g L^{-1} Peptone, 2 g L^{-1} Yeast extract, 2 g L^{-1} malt extract and 111 mM glucose). A sterile nylon net filter (100 μm Millipore #NY1H02500) was added to each well, and plates were grown in the dark at 28°C and 170 RPM for 24 hours. Following 24 hours growth, each well was washed twice with sterile dH_2O, before replacing with 4 mL of NP media (NP - 6.2 mM Putrescine di-hydrochloride, 22 mM KH_2PO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 85.6 mM NaCl, 116.8 mM sucrose, 108.6 mM glycerol v/v, pH 4.0). Growth continued under the same

conditions for additional 48 hours. The culture supernatant was collected and filtered (0.2 μM). The supernatant was diluted to 25% methanol and run on a Shimadzu prominence LC-20AD (Mandel) with 100 μL injection by a Shimadzu SIL-20A HT prominence auto sampler. Samples were run on a Restek Pinnacle DB C18 column (5um, 150x4.6mm #9414565) using a 22.5% isocratic MeOH:H_2O at 1 mL min^{-1} for 20 minutes. Trichothecene toxins were monitored by UV 220 ηm. Toxin amounts were quantified with a 15-ADON standard. Toxin amount was normalized with dried mycelial mass (μg toxin per μg tissue).

2.2.2.2 Perithecia Production & Quantification

To assess the mutant strains for sexual structures production, all strains were grown on carrot agar as described in Wang *et al*. (2013). Briefly, 400 g of baby carrots was autoclaved and pureed in a food processor in 900 mL dH_2O. Carrot puree was made to a final 1.8% agar concentration. Strains were grown on carrot agar before a single plug from each strain was transferred to fresh carrot agar and grown in constant UV light for 4 days. After 4 days, 1 mL of 2.5% Tween-60 was applied to each plate and plates were re-incubated under the same conditions until perithecia were visible after 4-5 days.

Quantification of ascospores was performed by adding 5 mL sterile water to each plate and incubating at room temperature and constant light overnight. The spores were collected and additional 10 mL sterile water was used to wash any residual spores from each plate. The spores were centrifuged at 5,000 RPM for 5 minutes and quantified by haemocytometer. This assay was repeated twice with 6-12 technical replicates for each strain.

2.2.2.3 Vegetative Growth

The amount of mycelial growth of each strain was measured by weight in the same nutrient poor conditions described in section 2.2.2.1. After 48 hours of nutrient poor growth, mycelial solids were collected and dried by desiccation before weighing. This was repeated for 2 biological replicates of 6 technical replicates each.

2.2.2.4 Pathogenicity Assay

Each strain was assessed for the ability to infect susceptible *Triticum aestivum* (cv. Roblin). Wheat heads were point inoculated at mid-anthesis with each strain at 100,000 spores mL^{-1}, in a volume of 10 µL (1,000 spores). Spores were inoculated between the palea and lemma of a single wheat spikelet. Plants were grown at 25˚C, 16 hour days and misting every hour for 30s. After 48 hours, misting was reduced to 30 s duration every 4 hours. Infection was scored by counting the number of visibly infected spikelets at days 4, 7, 9, 11 and 15.

2.3.1 Identification of C-5 Cytosine Methyltransferase in *Fusarium graminearum*

Two putative cytosine specific DNA methyltransferases were identified in *F. graminearum*. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis indicated FGSG_10766 and FGSG_08648 are homologous with two well characterized DMTs in *N. crassa*: NCU02247 (DIM-2) and NCU02034 (RID). FGSG_10766 shared 49% (e value: 0.00) sequence identity with DIM-2, and FGSG_08648 shared 35% (e value: $4.0x10^{-93}$) amino acid sequence identity with RID. Reciprocal BLASTs yielded similar results. Phylogenetic analysis of 35 DNA methyltransferases from 29 eukaryotic species (16 fungi, 12 mammals, 1 plant) and 1 prokaryotic species (*E. coli*) was performed to understand the evolutionary origin of DNA methyltransferases identified in *F. graminearum*. Sequence alignment and maximum likelihood analysis resulted in 4 clades: DNMT1 to DNMT4 (**Figure 3**).

The DNMT1 clade was further divided into DNMT1 from higher eukaryotes (plants and mammals, green) and DNMT1 from fungal species (Fungal DNMT1, red; **Figure 3**). The FGSG_10766 from *F. graminearum* clustered with the fungal DNMT1 clade. It appears most closely related to DIM-2 from the parasitic fungus *Beauvaria bassiana* (**Figure 3**). The DNMT4 clade is comprised entirely of fungal species, including the *F. graminearum* FGSG_08648. This DMT was most closely related to RID from *Neurospora* spp. (DNMT4, purple; **Figure 3**). Each grouping was supported strongly by bootstrap analysis, and bootstrap values above 50 can be seen at nodes on the **Figure 3**. For example, the fungal DNMT4 clade is supported by 99% of bootstrap

samples; similar confidence is observed in the separation of the DNMT1 clade, by 100% of bootstraps.

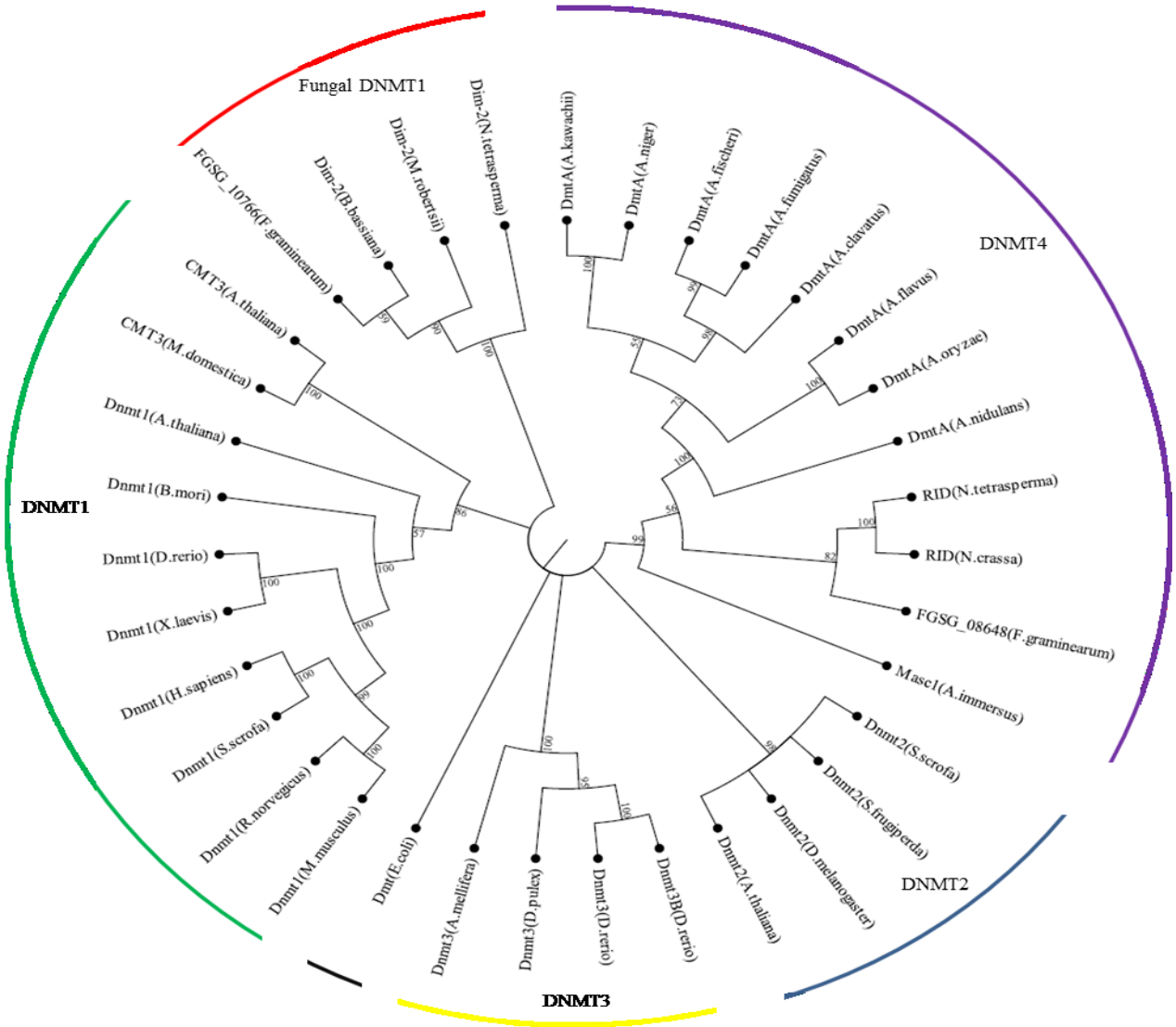


Figure 3: DNA methyltransferases from *F. graminearum*: FGSG_10766 and FGSG_08648. Five distinct groups of methyltransferases, DNMT1 (Plant and animal: Green; Fungal: Red), DNMT2 (Blue), DNMT3 (Yellow), DNMT4 (Purple) are indicated. A bacterial DNA methyltransferases was used as an outgroup (Black).

The placement of *F. graminearum* methyltransferases in the phylogenetic tree was supported by analysis of domain architecture of DMTs (**Figure 4**). The DNMT1 from higher eukaryotes (e.g. plants and mammals) displayed more complex domain architecture with the presence of DMAP1 binding domain, C-5 cytosine methyltransferase replication foci domain and ADD domain, in addition to the main methyltransferase and bromo-adjacent homology domains (**Figure 4**). The majority of these domains are absent from the fungal DNMT1 clade, which only harbor the main C-5 methyltransferase domain and bromo-adjacent homology domains (**Figure 4**).

The DNMT4 clade displayed simple domain architecture, with a single C-5 methyltransferase domain. The exception is Masc1 from *A. immersus,* which also contains the bromo-adjacent homology domain (**Figure 4**). Based on domain architecture, the DNMT4 clade appeared most similar to the DNMT2 clade, as well as the outgroup *E. coli* clade. Both fungal DNMT groups have reduced domain complexity compared to the expanded domains found in higher eukaryotes, specifically the DNMT1 and DNMT3 clades. The DNMT3 clade has the highest domain complexity, including calponin-homology domains, PWWP (Proline – Tryptophan – Tryptophan – Proline) domains, Zinc finger domains and chromo-domains are absent in both fungal clades (**Figure 4**).

A whole genome transcription profile of *F. graminearum* showed that the *DNMTs* are constitutively expressed at low levels in both nutrient rich (NR) and nutrient poor (NP) conditions (~60 reads for *FGSG_10766* and <50 reads for *FGSG_08648* by RNA sequencing, K. Shostak, personal communication).

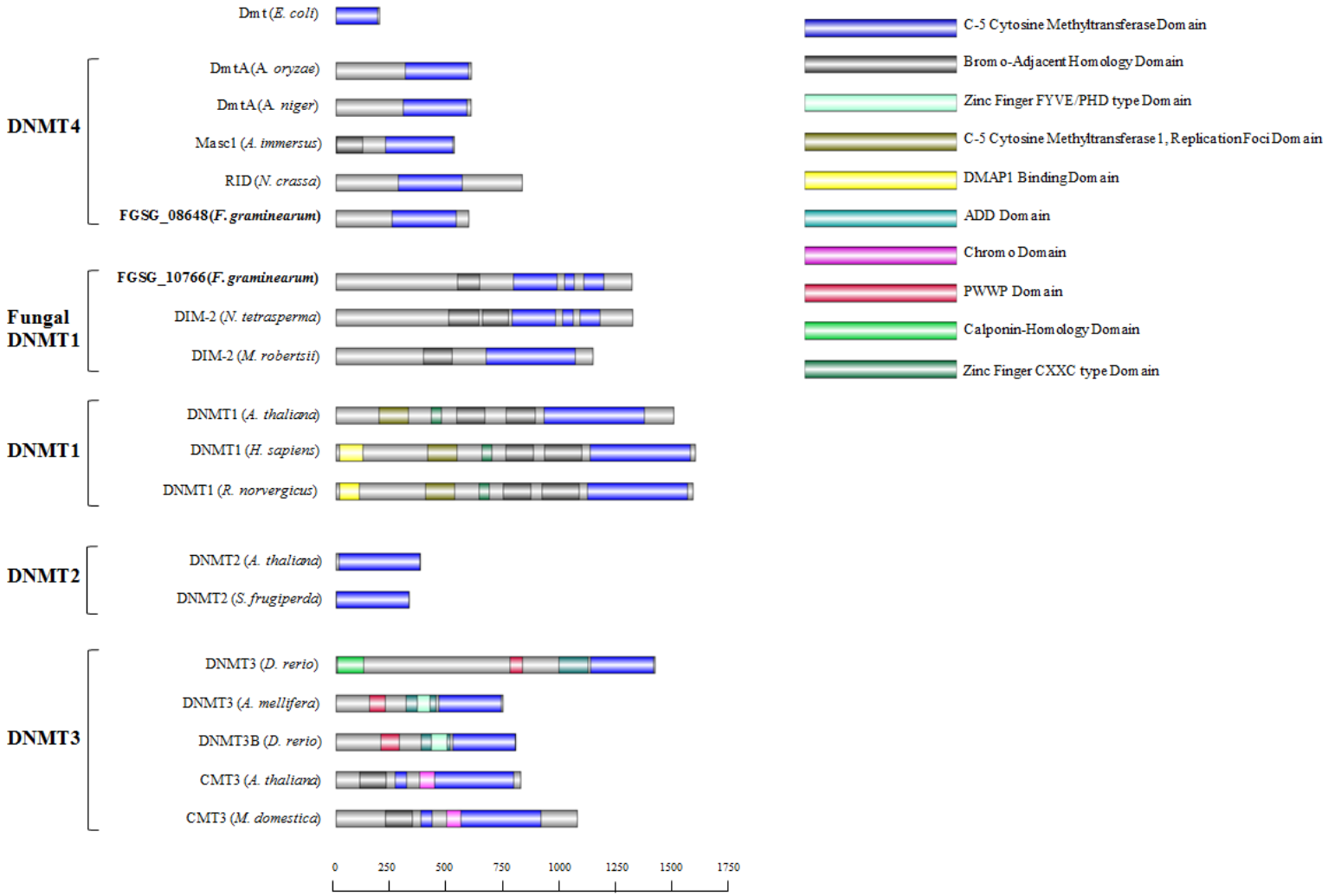


Figure 4: Domain architecture of DNA methyltransferases in *F. graminearum* are shown in bold. Protein domains were visualized using the Illustrator of Biological Sequences (IBS). Scale represents amino acid number.

2.3.2 Vegetative growth is regulated by DNA Methyltransferases in *F. graminearum*

Vegetative growth was measured after 48 hours in nutrient poor liquid culture for each mutant: Δ*dnmt1*, Δ*dnmt4*, Δ*dnmt1*/Δ*dnmt4*, Δ*dnmt4*:*DNMT4*. Although no difference in growth was observed for the single deletion strains, a significant decrease was observed for the double deletion strain, compared to the *WT* (**Figure 5**).

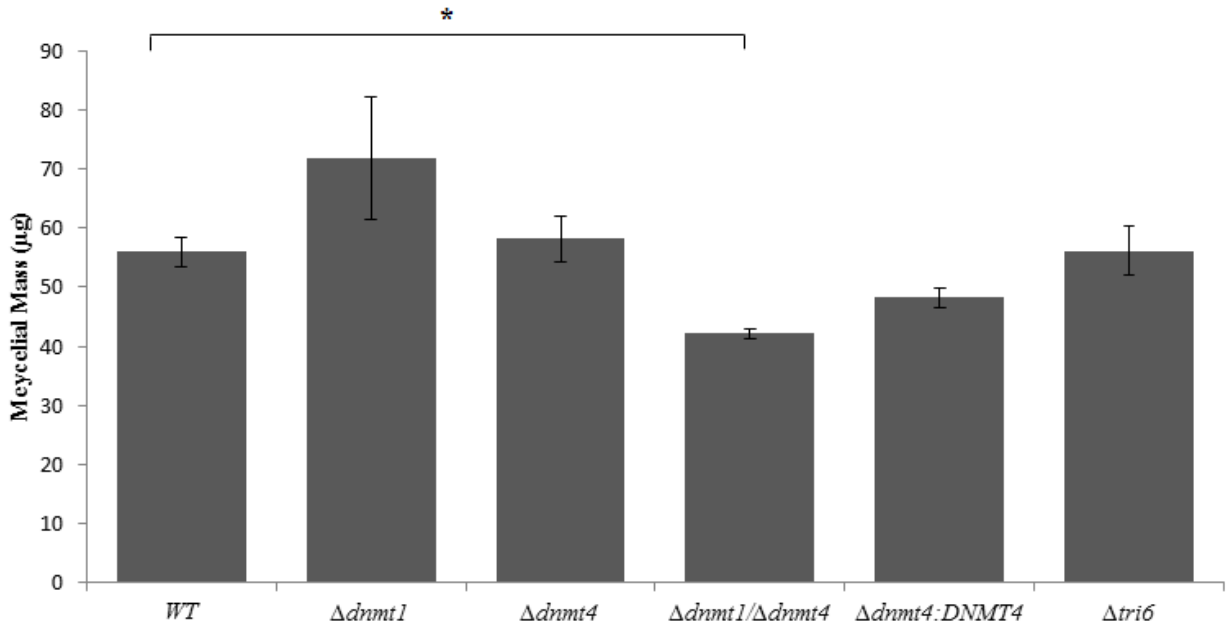


Figure 5: DNA methyltransferase mutant Δ*dnmt1/*Δ*dnmt4* has altered growth. Sample mass (µg) was compared to *WT* using a two-tailed Student's *t* test for significance ($\alpha = 0.001$), represented by a single asterisk (*). Error bars represent standard error of the mean.

2.3.2 DNA methyltransferases in *F. graminearum* have a role in SM production

The ability of mutant strains to produce the fungal mycotoxin 15-ADON was assessed by high performance liquid chromatography (HPLC). The single mutant strains were able to produce 15-ADON in similar amount to the *WT* strain, while the double mutant produced 1.5-fold more 15-ADON compared to the *WT* strain (**Figure 6**). Additionally, HPLC analysis also identified a novel metabolite at the retention time of 6.6 minutes (**Figure 7**). This SM metabolite was present at negligible levels in the *WT* and both single mutant strains and accumulated to 8.4-fold more in the double deletion strain (**Figure 7**). The identity of this novel metabolite is under investigation.

The reddish pigment aurofusarin, produced by *F. graminearum* is the product of polyketide biosynthesis gene *PKS12* (Frandsen *et al.* 2006). When grown on PDA medium, *F. graminearum* characteristically produces aurofusarin. Mutant strains were grown (>10 days) on PDA media and were qualitatively examined for pigmentation. All strains were able to develop a reddish pigmentation, however; mutant strains lacking *DNMT1* in some instances did not produce this reddish pigment. This was observed in 33% of cases for the double deletion strain, and 16% of cases for the single deletion of *DNMT1* (n=6; **Figure 8**). Indeed, even in the Δ*dnmt4* strain, we observed slight decreases in the amount of red pigment compared to *WT* (**Figure 8C**).

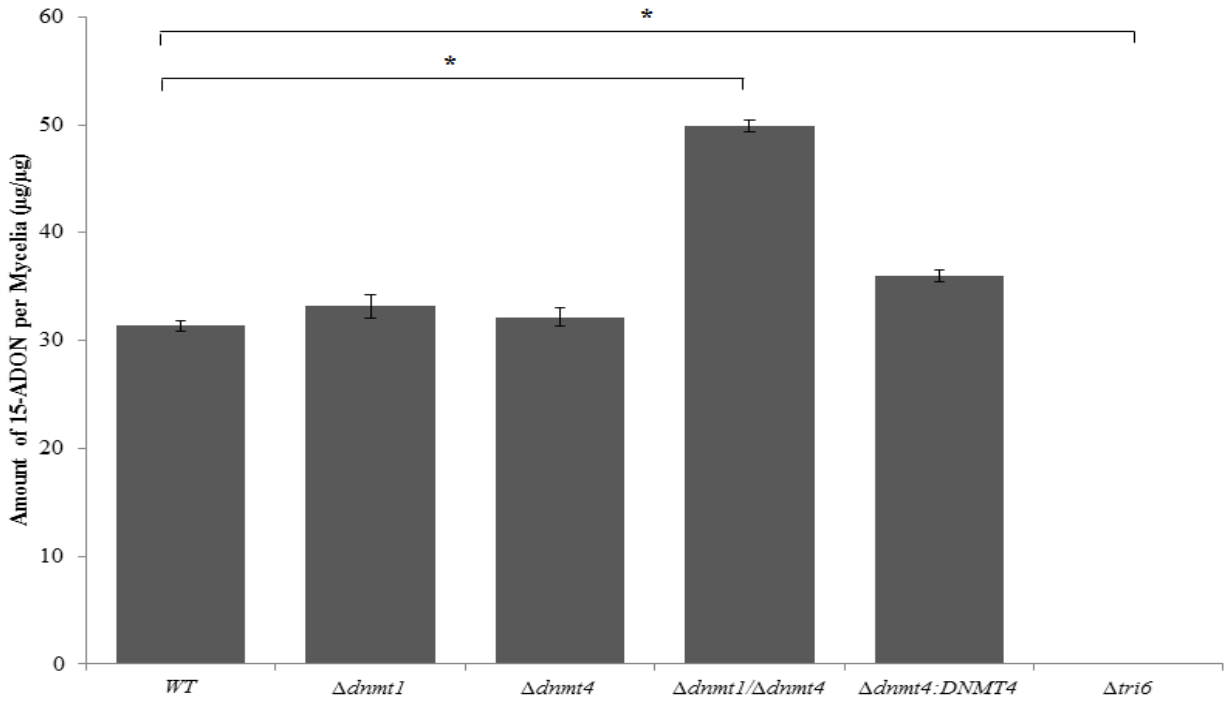
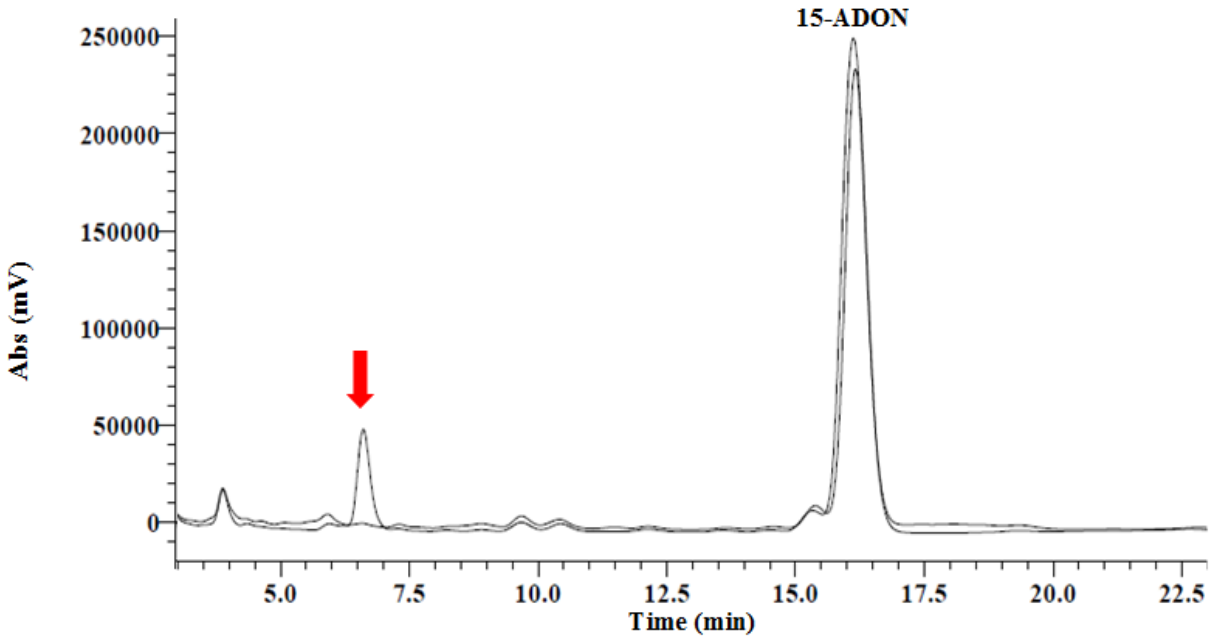


Figure 6: DNA methyltransferases are involved in the regulation of mycotoxin deoxynivalenol in *F. graminearum*. Toxin is represented on the Y-axis as amount of 15-acetyl-DON (15-ADON; µg) per amount of mycelia (µg). Results are pooled across 6 technical replicates each from two biological replicates. Error bars represent standard error of the mean, and significance ($\alpha = 0.001$) is represented by a single asterisk (*), as determined by a two-tailed Student's *t* test in comparison to *WT*.



Strain	Retention Time (min)	Peak Area per Mycelial Mass (Area/µg)	Fold Change
WT	6.6	1629	8.4
Δdnmt1/Δdnmt4	6.6	13678	

Figure 7: DNA methyltransferase double deletion mutant produces novel metabolites, shown by the red arrow. Average peak area was taken from 6 biological replicates across two technical replicates, and normalized to mycelial mass (µg), resulting in a significant difference using a two-tailed Student's *t* test for significance ($\alpha = 0.001$).

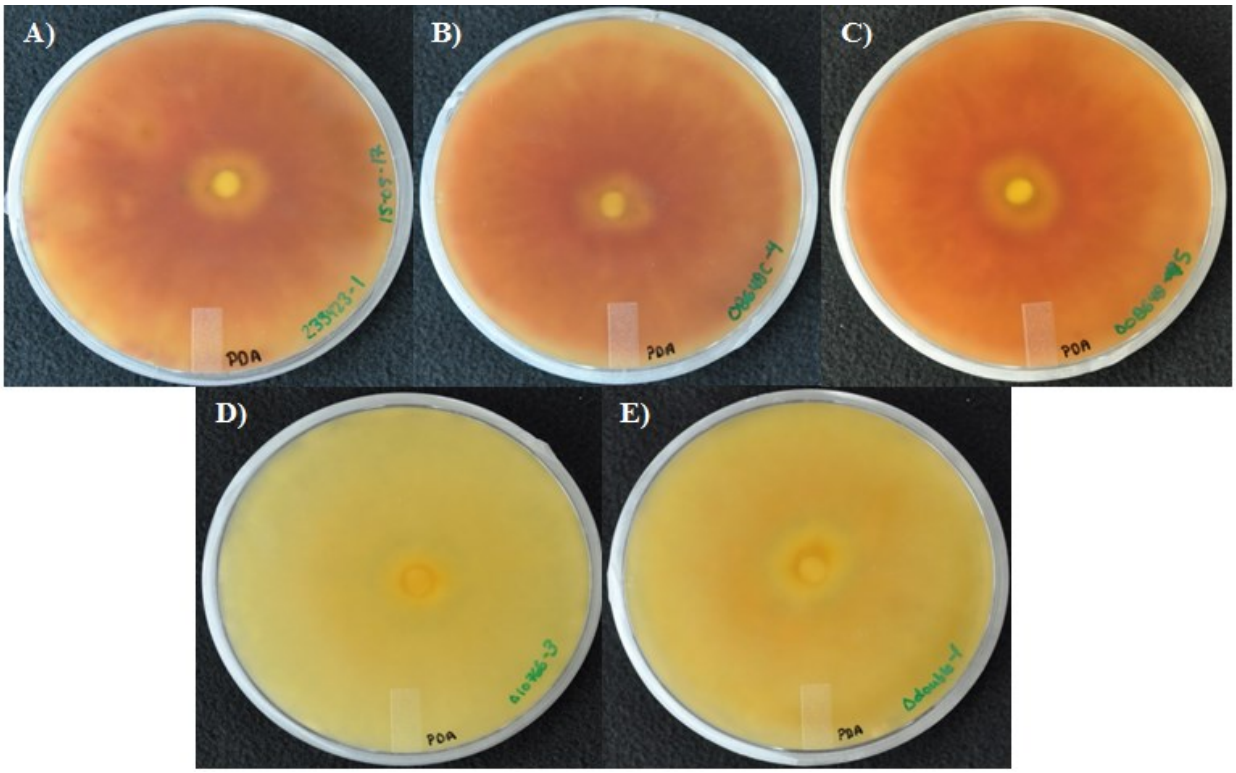


Figure 8: *DNMT* mutants display altered pigmentation. A) wild-type *F. graminearum* B) Δ*dnmt4* C) Complemented strain of Δ*dnmt4:DNMT4* D) Deletion mutant of Δ*dnmt1* E) Double deletion mutant strain Δ*dnmt1*/Δ*dnmt4*.

2.3.3 DNA methyltransferases affect ascospore production in *F. graminearum*

Mutant strains were examined for the ability to produce perithecia and ascospores on carrot agar. All mutant strains were able to produce perithecia; however the single mutant (Δ*dnmt1*) and the double mutant (Δ*dnmt1*/Δ*dnmt4*) produced significantly less ascospores than *wild-type* (**Figure 9**). No significant difference was observed for the mutant strain Δ*dnmt4* (**Figure 9**). All strains produced viable ascospores capable of germination on PDA media (data not shown).

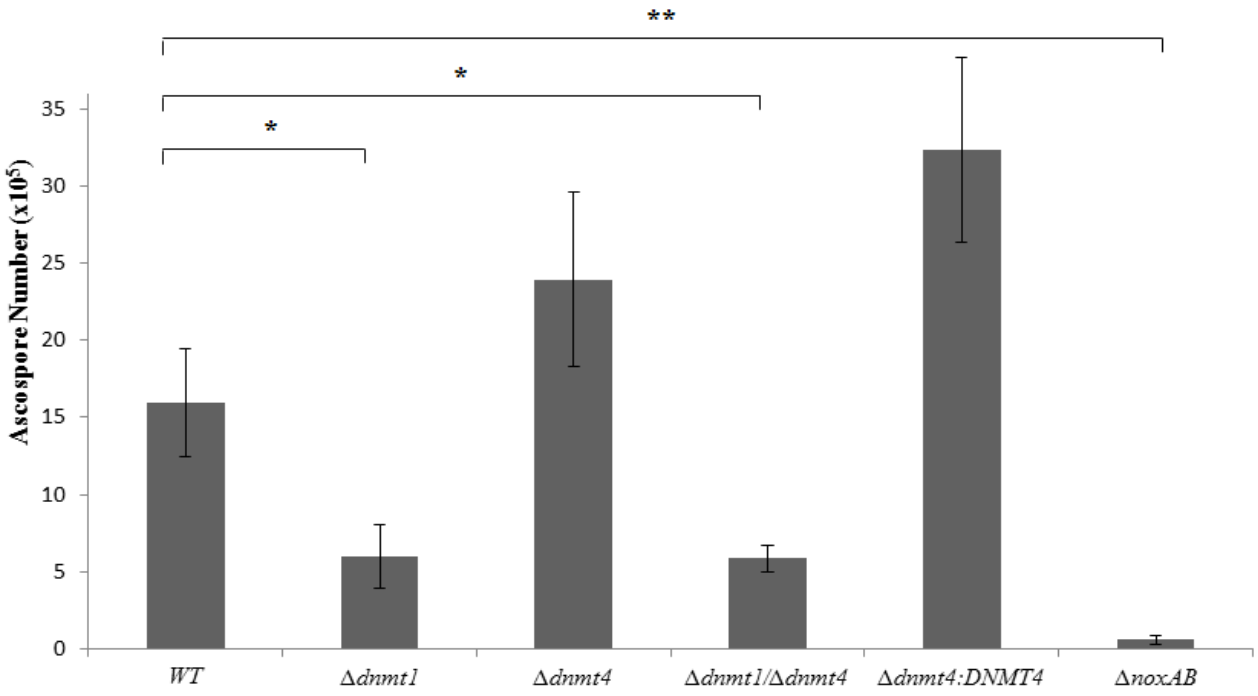


Figure 9: Ascospore production is altered in DNA methyltransferase mutants. Methyltransferase mutant strains (x-axis) were examined for the ability to produce ascospores (y-axis). Results represent 1 biological replicate with 6-9 technical replicates; error bars represent standard error of the mean. A single asterisk (*) represents a significance of $0.05 \geq P$ value ≥ 0.01, while two asterisks (**) represents a significance of P value ≤ 0.01, compared to *WT*, by two-tailed Students *t* test.

2.3.4 *FgDNMT1* and *FgDNMT4* in *F. graminearum* have a role in virulence

Both single and double mutant strains were assessed for their ability to infect susceptible wheat cultivar 'Roblin'. Spores from *WT* and the *DMT* mutant *F. graminearum* strains were inoculated and monitored for disease symptoms. After 15 days post inoculation (DPI), *WT* strain was able to infect the majority of the wheat heads (93%). Both the single deletion mutants had a modest decrease in virulence (~ 10% reduction) compared to *WT* strain (**Figure 10**). On the other hand, the double deletion strain was able to infect on average only 65% of the wheat heads ($\alpha < 0.001$) (**Figure 10**). This suggests that *FgDNMT1* and *FgDNMT4* together contribute to the overall pathogenicity of the fungus.

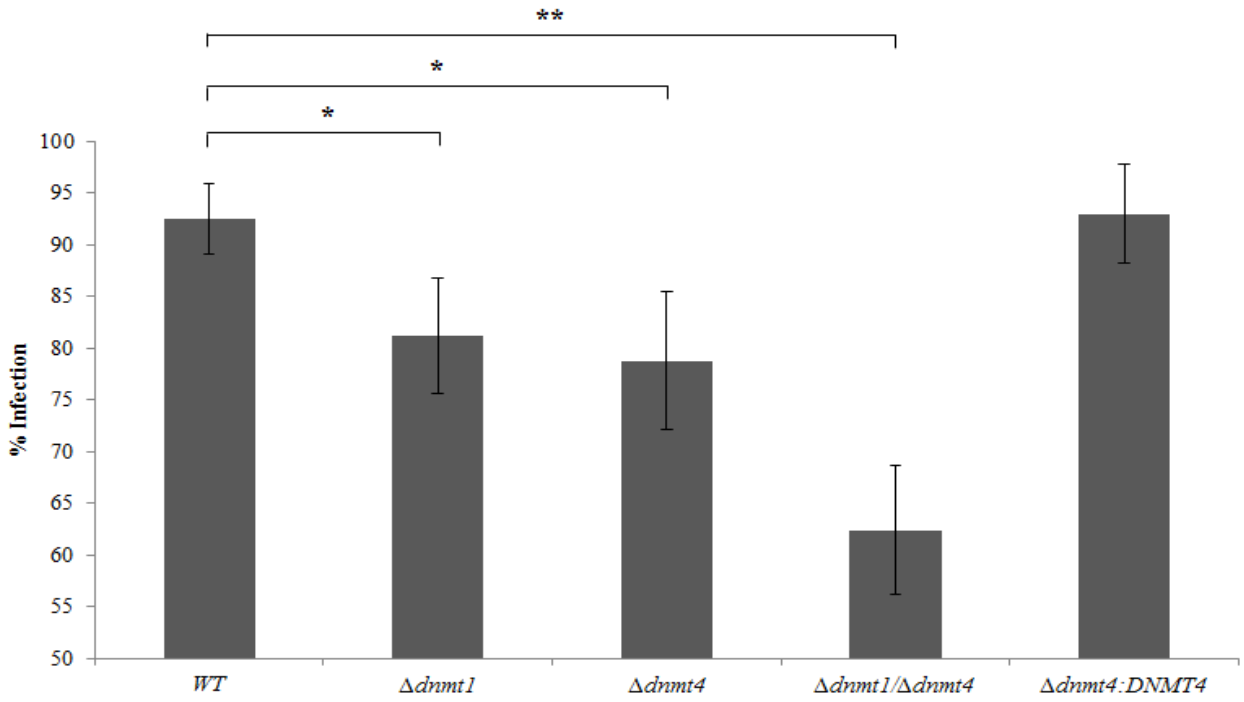


Figure 10: *F.* graminearum DNMT mutants affect disease outcomes in wheat. Wheat heads were point inoculated with 1,000 conidia of each strain: *WT* (n = 33), Δ*dnmt1* (n = 30), Δ*dnmt4* (n = 29), Δ*dnmt4*:Δ*dnmt1* (n = 45), Δ*dnmt4*:*DNMT4* (n = 18) where n represents the number of wheat heads. Results are presented as percent spikelets with disease symptoms present at 15 days post-inoculation (DPI). Error bars reflect standard error of the mean. A single asterisk (*) represents a significance of $0.05 \geq P$ value ≥ 0.01, while two asterisks (**) represents a significance of P value ≤ 0.01, compared to WT by two-tailed Students *t* test.

2.4.1 DNA Methyltransferases in *F. graminearum*

This study investigated the role of DNA methylation in the phytopathogen *F. graminearum* in fungal development and pathogenicity. We identified two putative DNA methyltransferases, FGSG_10766 and FGSG_08648, by domain and homology searches to closely related fungi *N. crassa* DNA methyltransferases.

Our phylogenetic analysis identified FGSG_10766 to be closely related to DIM-2 (deficient-in-methylation) sub-family DMTs (**Figure 3**). These methyltransferases are typically grouped with the DNMT1 family, however, DIM-2 appears to be divergent from DNMT1 suggesting these methyltransferases may be functionally different in addition to being structurally different to other DNMT1 family members (**Figure 3**; Ponger and Li 2015; Kouzminova and Selker 2001). We observed differences in domain architecture in *Fg*DNMT1 in relation to mammalian or plant DNMT1s, such as reduced domain complexity (**Figure 4**). As such, the role of DNMT1 in *F. graminearum* requires further investigation.

Our phylogenetic tree also indicated that FGSG_08648 is closely related to fungal DNMT4 family, and may play a similar role to RID (repeat-induce-deficient) in *N. crassa* and *N. tetrasperma* (**Figure 3**). The RID DMT is annotated as C-5 DNA methyltransferase containing the required domains for deposition of 5mC (**Figure 4**), however, to date, little evidence exists in fungi that these enzymes are responsible for methylation in any capacity, either *de novo* or in maintenance (Freitag *et al.* 2002). These DMTs, instead, have been found necessary for repeat induced point mutations (RIP)

(Freitag *et al.* 2002). The RIP mechanism inactivates genes in repetitive DNA sequences during sexual development, suggesting a possible role for *DNMT4* in sexual development. Indeed, studies have often linked DNA methyltransferases to the sexual stage in fungi; for example, a DNMT4 family member DmtA in *A. nidulans* has been shown to be required for early sexual development as well as for formation of viable ascospores (Lee *et al.* 2008).

2.4.2 DNA Methyltransferases Affect Fungal Development

We studied the ability of *FgDNMT* mutant strains to produce sexual structures called perithecia, and quantified the number of ascospores produced by each strain (**Figure 9**). Interestingly, we observed differences in ascospore production in the *dnmt1* deletion strains (Δ*dnmt1* and Δ*dnmt1*/Δ*dnmt4*), rather than the *dnmt4* deletion strain. This result was unexpected, as deletion of *dmtA* in *A. nidulans* (*AnDNMT4*) affected production of sexual spores (Lee *et al*. 2008). We observed a similar decrease in the double mutant strain, suggesting the mechanism of action of *FgDNMT1* is separate from *FgDNMT4*. In this case, it is likely that *FgDNMT1* has a role in sexual development in *F. graminearum*, through yet unknown mechanisms.

Unlike sexual spore development, both *DNMT1* and *DNMT4* have been implicated in vegetative growth development (Jeon *et al.* 2015). We therefore assessed *FgDNMT* mutant strains for vegetative growth. We observed that both DNA methyltransferases are not necessary for normal vegetative growth, as only the double deletion strain demonstrated reduced growth (**Figure 5**). These results may also suggest that DNA methylation in *F. graminearum* is, in part, involved in the regulation of growth

in response to changes in nutrient conditions and availability. It would be interesting to assess the role *FgDMTs* with respect to various carbon and nitrogen sources.

2.4.3 DNA Methyltransferases and Secondary Metabolism

A study with a histone methyltransferase gene *KMT6* was the first to show a direct link between methyltransferases and SM biosynthesis in *F. graminearum* (Connolly *et al.* 2013). An indirect link between DNA methyltransferases and SM production was established in *F. graminearum* with a study of *HP1,* a gene required for the recruitment of DMTs (Dominguez *et al*. 2012). In fact, deletion of this gene resulted in changes in the accumulation of 15-ADON and aurofusarin production in culture (Dominguez *et al*. 2012). Genetic analysis performed in this thesis is the first to show a direct link between *FgDNMTs* and SM production (**Figures 6, 7 and 8**). It is conceivable that alterations in chromatin structure mediated by HP1 may influence the activity of *Fg*DMTs and together be responsible for the regulation of genes involved in 15-ADON biosynthesis and other SMs.

In contrast to findings in *A. flavus* where a single deletion of *DNMT* (Δ*dmtA*) was sufficient to alter the production of aflatoxin, both DMTs are required to affect SM production in *F. graminearum* (Yang *et al.* 2016). Although these two *Fg*DMTs exhibit similar structural properties to other fungal species, differences may exist in their regulation, including interaction with co-enzymes or histone proteins and other epigenetic marks. For example, DNA histone methyltransferase and associated complex proteins DIM-5,-7,-9, CUL4, DDB1 (DIM-8) and HP1 (heterochromatin protein 1) are known to contribute to DNA methylation (Freitag *et al.* 2004; Lewis *et al.* 2010b). Thus, in addition

to HP1, other proteins associated with chromatin remodelling may also influence the activity of DMTs.

Aberrant DNA methylation patterns resulting from the deletion of both DMTs could be responsible for these changes in SM production, possibly through changes in gene expression (by chromatin remodelling or interfering with transcription machinery) of important SM genes, such as those involved in the trichothecene gene cluster for toxin production.

2.4.4 DNA Methyltransferases have a Role in Virulence

Contrasting findings have also been presented with respect to the role of DMTs in pathogen virulence. For example, DNA methylation was found to be dispensable for pathogenicity in the rice blast fungus *M. oryzae*, while *DmtA* contributed to virulence in *A. flavus* (Jeon *et al.* 2015; Yang *et al.* 2016). Similar to what we observed with the regulation of SMs, changes in *F. graminearum* pathogenicity are only observed upon deletion of both DMTs (**Figure 10**). However, these results also underscore the complexity of DMT function in *F. graminearum*. For example, the double mutant showed an increase in 15-ADON production, which theoretically should lead to increase in the spread of the pathogen. Paradoxically, we observed a decrease of virulence in the double mutant strain (**Figure 10**). This apparent contradictory result is not without precedent. We have shown previously that a strain lacking the enzyme NADPH oxidase (NOX) is capable of producing 15-DON in culture, but is non-virulent (Wang *et al.* 2013). It's conceivable that similar to NOX, DMTs regulate genes involved in the initial infection process and deletion of both DMTs compromise the pathogen's ability to infect.

Furthermore, neither the normal production of 15-ADON, as seen in the NADPH mutant strain nor the overproduction of 15-ADON, as observed in the double *DNMT* deletion strain can overcome the pathogen's inability to infect the host. Genome analyses have identified candidate virulence genes and it would be interesting to see if any of these candidate virulence genes undergo methylation changes (Walkowiak *et al*. 2016).

In summary, we have identified numerous phenotypic differences in the DMT deletion strains, both in fungal development and pathogenicity. The exact mechanisms for these observations are unknown, however; we suggest DNA methylation may be essential in regulation of both development and pathogenicity in *F. graminearum*.

Chapter 3: DNA Methylome Analysis of *F. graminearum*

Chapter 3 – Introduction

3.1.1 Fungal DNA Methylation

DNA methylation is an epigenetic mark found in a wide diversity of eukaryotic species (Zamach *et al.* 2010). Specifically, methylation of the 5th position of cytosine bases (5mC) provides critical epigenetic regulation of not only transposable elements (TEs) and repetitive sequences, but also in regulating gene expression (Zamach *et al.* 2010). Aberrant DNA methylation has been implicated in a number of human diseases, such as cancer (Robertson 2005). DNA methylation occurs on all three genomic contexts, CG, CHG and CHH (H = A,C,T), although in varying frequencies in different organisms (Krueger and Andrews 2011; Gehring and Henikoff 2007). For example, DNA methylation in higher eukaryotes occurs mainly on cytosine in the CG context, while in plants, methylation occurs frequently at CG and CHG contexts, but also occurs in the CHH context (Li and Zhang 2014; Gehring and Henikoff 2007; Vanyushin 2006). In fungi, DNA methylation is also found across all three contexts. For example, DNA methylation in *M. oryzae* occurs predominantly in the CG and CHH contexts (Jeon *et al.* 2015). DNA methylation is also heterogeneous within a population, and thus each cytosine locus will be present in both methylated and non-methylated cytosine forms (Qu *et al.* 2016).

3.1.2 Techniques for the Study of DNA Methylation

Numerous methods have been developed to aid in the study of methylation and methylation patterns (Kurdyukov and Bullock 2016). Choosing a method for the study of 5mC depends on the *a priori* knowledge of methylation in the system. For example, cases in which candidate genes or regions of interest are known, smaller scale analysis can be performed, including enzyme based digestion, methylation specific PCR (MSP), pyrosequencing, and high resolution melt (HRM), among others (Bird and Southern 1978; Herman *et al.* 1996; Tost and Gut 2007; Wojdacz and Dobrovic 2007; Zamach *et al.* 2016). Enzyme based digestion relies on restriction enzymes such as *Hpa*II, BstUI, *Not*I, and *Sma*I, which are methylation sensitive restriction enzymes (MREs), able to cleave non-methylated DNA but not methylated DNA (Bird and Southern 1978; Yong *et al.* 2016). Many techniques make use of a sodium bisulfite chemical treatment, which chemically converts non-methylation cytosine bases into uracil and then subsequently thymine by PCR cycling; methylated cytosines are protected from this conversion (Patterson *et al*. 2011). HRM techniques make use of differences accrued in bisulfite treatment (C → T) to result in dramatically different melting curves following PCR amplification, as more heavily methylated sequences will retain more cytosine than sequences that have less 5mC, altering the melting profile (Wojdacz and Dobrovic 2007). Similarly, one of the more widely utilized techniques for individual cytosine methylation mapping is MSP (Herman *et al.* 1996; Wojdacz and Dobrovic 2007). DNA is bisulfite converted and PCR amplified using primers specific for methylated DNA (no C → T conversion in the primer region). Amplification indicates cytosine loci within the primer

regions are methylated, and band intensity can be directly correlated to methylation level within a population (Herman *et al.* 1996).

Techniques that do not require prior knowledge of candidate genes or positions of interest provide genome wide information on methylation states include techniques such as ELISA, HPLC-UV, mass spectroscopy (MS) and whole genome bisulfite sequencing (WGBS), providing varying levels of sensitivity and detail. For example, ELISA based methods quantify genome wide methylation levels by measuring colorimetric or fluorometric signals corresponding to the binding of 5mC to specific monoclonal antibodies (Kurdyukov and Bullock 2016). ELISA based assays require a high level of methylation to accurately detect methylation signals (Kurdyukov and Bullock 2016). HPLC-UV monitors differences in retention times between 5mC and cytosine bases to quantify relative levels of methylation (Johnston *et al.* 2005). HPLC-UV methods, similar to ELISA, suffer from limited sensitivity, especially for samples with low levels of methylation; as such, this technique is often used in plant or animal studies (Johnston *et al.* 2005; Armstrong *et al.* 2011). MS based techniques offer increase sensitivity for low methylation levels. MS is reliant on the fact that 5mC has a different mass/change (m/*z*) ratio compared to unmodified cytosine (Kurdyukov and Bullock 2016). Indeed, MS based analysis has been validated for the detection of DNA methylation levels as low as 0.05% (Song *et al*. 2005).

The gold standard in the study of DNA methylation patterns is WGBS, owing to excellent sensitivity and the ability to offer information on methylation states at the individual cytosine level (Yong *et al.* 2016; Kurdyukov and Bullock 2016). WGBS is a combination of sodium bisulfite conversion, followed by whole genome sequencing

(Yong *et al.* 2016). Whole genome bisulfite sequencing relies on the conversion of genomic DNA by sodium bisulfite. A product of this conversion is decreased read complexity, as non-methylated cytosine are converted to uracil and subsequently thymine during PCR amplification, resulting in reads with high T or A frequencies on the forward/reverse strands, respectively (Yong *et al.* 2016; Kurdyukov and Bullock 2016). The treatment of DNA with sodium bisulfite can also lead to DNA fragmentation which, in addition to the reduced read complexity, generates short, low complexity reads and results in poor read mapping efficiencies (Kurdyukov and Bullock 2016). Numerous software have been developed to tackle the issue of aligning WGBS reads, including BSMAP and Bismark (Xi and Li 2009; Krueger and Andrews 2011). Bismark provides the best trade-off between speed, coverage and accuracy. Bismark is also one of the first mapping software to consider all three cytosine contexts, instead of just the CG context (Kunde-Ramamoorthy *et al.* 2014; Tran *et al.* 2014). Bismark uses the short read aligner Bowtie2 (Langmead *et al.* 2009). A novel WGBS alignment package has also recently been released as part of the CLC-Genomics Workbench software. Benchmarking test done by Qiagen suggest the CLC-Genomics Workbench package is able to correctly place upwards of 30% more reads, in a shorter time frame than Bismark (Qiagen 2017). Due to the limitations of WGBS, including reduced complexity, DNA degradation and fragmentation, higher mapping efficiencies may result in higher coverage and thus increased confidence in sequencing results.

In addition to providing information on methylation levels (# methylated reads covering one 5mC locus / # total reads covering that locus) and methylation percentage (# methylated cytosine genome wide / # total cytosine genome wide), WGBS is able to

provide information on differential methylation between conditions. For example, methylation of a specific cytosine locus can be compared, and differences that exist between conditions can be called as differentially methylated cytosine (DMC). Differences between regions of cytosine can be called as differentially methylated regions (DMRs). A number of programs and packages are available for the statistical determination of both DMCs and DMRs, including MethylKit (Akalin *et al.* 2012).

This study examined the methylation states of *F. graminearum* across two environmental conditions: nutrient rich (NR) and nutrient poor (NP) by WGBS.

3.2.1 Fungal Strains

A 15-ADON producing F. *graminearum* strain DAOM233423 (*WT*) was used to query DNA methylation. The fungal strain was obtained from the Canadian Collection of Fungal Cultures (ORDC, AAFC). Experiments were initiated using freshly developed macroconidia and generated in carboxymethylcellulose (CMC) medium (Walkowiak *et al.* 2015). For longer term storage, conidia were suspended in 15% glycerol and stored at -80˚C. Frozen stocks were used to maintain fresh spores as the starting inoculum for each experiment. Strains were only propagated through a maximum of two generations.

3.2.2 Growth and Isolation of Fungal DNA and Whole Genome Bisulfite Sequencing

F. graminearum spores were inoculated into 4 mL rich medium (NR – 56 mM NH_4Cl, 8.1 mM $MgSO_4 \cdot 7H_2O$, 0.23 mM $FeSO_4 \cdot 7H_2O$, 14.7 mM KH_2PO_4, 2 g L^{-1} peptone, 2 g L^{-1} yeast extract, 2 g L^{-1} malt extract and 111 mM glucose) containing a 100 µm nylon net filter (Millipore #NY1H) at 5,000 spores mL^{-1} in 6-well sterile culture plates. Cultures were incubated at 28 ˚C and 170 RPM in darkness for 24 hours with constant shaking in a New Brunswick Scientific Innova44 Shaker. After 24 hours, triplicate samples of mycelia were removed from culture, washed briefly with dH_2O and flash frozen in liquid N_2. A second set of samples were grown as above in NR medium, and mycelia were also washed and transferred to 4 mL of a nutrient poor (NP - 6.2 mM putrescine dihydrochloride, 22 mM KH_2PO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 85.6 mM NaCl,

116.8 mM sucrose, 108.6 mM glycerol v/v, pH 4.0) and incubated for an additional 6 hours under the same conditions. DNA was extracted from frozen mycelia using the E.Z.N.A Fungal DNA Mini Kit (Omega Bio-tek #D3390), and quantified by Nano-drop (ND-1000 Spectrophotometer). DNA quality was assessed on a 1% agarose gel with GelRed dye.

Duplicate samples for 24 hour NR and 6 hour NP were assessed for methylation by WGBS at the Centre for the Analysis of Genome Evolution & Function (CAGEF, University of Toronto). Library preparation included the shearing of genomic DNA to ~300 bp size (Covaris S2) and size selection, followed by end repair, dA-tailing and adaptor ligation (New England Bio-labs NEBNext DNA Library Prep Master Mix Set for Illuminia #E6040) was performed using NEB methylated adaptors (#E7535), substituted for standard Illuminia paired end adaptors following manufacturer's instructions. Sodium bisulfite conversion was performed using the Qiagen EpiTect kit and protocol (#59104), followed by clean up and amplification of converted DNA following NEB methylated adaptor protocol. Amplification used NEB EpiMark Hot Start Taq polymerase (#M0490) and PCR was cycled 13 times. Libraries were quantified using the Qubit fluorometer (Life Technologies) and pooled into equal quantities. The final library was assessed for quality on a Bioanalyzer 2100 using the DNA1000 chip (Agilent). The pooled library was sequenced 2x on an Illumina Miseq at 150 base paired end (PE) reads. phiX Lambda DNA was incorporated into the library at 10% as an internal control.

3.2.3 Analysis by CLC-Genomics Workbench

Paired end sample reads were imported into CLC-Genomics Workbench (Qiagen Bioinformatics v9.5.3) and were first trimmed in CLC-Genomics Workbench for quality, adaptor/primer contamination and length. Reads trimmed for quality score limit of 0.05 with a maximum of 2 ambiguous nucleotides. Methylated adaptors and primer sequences provided by Illuminia and NEBNext were trimmed from both strands. Reads with a post-trim length of <30 nucleotides were discarded. The reference for the *WT* fungal strain (NCBI LAJZ01) was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/assembly/GCA_000966635.1). Bisulfite sequencing reads were mapped to the reference genomes using the Bisulfite Sequencing Plug-in (Qiagen Aarhus v 1.1.1) tool 'Map Bisulfite Reads to Reference' using the default parameters. Uniquely mapped reads were assessed for methylation calls using the Bisulfite Sequencing Plug-in tool 'Call Methylation Levels' to determine methylation levels in 3 contexts: CpG, CHG and CHH. The first and last 5 bases of a read were ignored to avoid methylation bias common towards the beginning and end of a read. Methylated cytosine contexts were confirmed in the reads by the software to avoid false positive calls resulting from variants.

A binomial model was used to remove methylation calls due to incomplete bisulfite conversion or sequencing error (Garg *et al.* 2015; Schultz *et al.* 2012; Liu *et al.* 2012). The following formula:

$$C_n^x p^x (1-p)^{n-x} < q$$

Equation 1: Binomial Distribution for 5mC methylation calls.

where ‘n’ is the number of methylated read calls, ‘x’ is the number of total reads (sequencing depth) for a specific cytosine, ‘*p*’ is the false-positive rate (1 - Conversion rate) and ‘q’ is the significance threshold (Liu *et al.* 2012). Methylation sites supported by binomial testing ($\alpha = 0.05$) were used for subsequent downstream analysis. A minimum read depth of 2 was also required.

Methylcytosine sites were organized into 10 kb bins and average methylation percentage was calculated (# 5mC sites / total cytosine sites *100) for both NR and NP conditions. Methylation percentage in each bin for NP conditions was subtracted from the methylation percentage in each bin for NR conditions; this resulted in a measure of methylation difference between each bin. Secondary metabolite gene cluster locations were obtained from Sieber *et al.* (2014). The program MethylKit (v1.2.0) in the R-package Bioconductor was used to determine similarity between replicates, by means of the Pearson Correlation Coefficient, to generate dendrograms of the sample (Akalin *et al*. 2012). MethylKit was also used to search for differentially methylated regions (DMRs); regions defined in 1 kb blocks as the number of 5mC in one nutrient condition was significantly different from the other condition. Significance was assessed through Fisher’s Exact Test in order to generate *P*-values (Akalin *et al*. 2012). The 5mC loci that were overlapping between BRs of a nutrient condition or between nutrient conditions were examined, and their position relative to genic features such as gene promoters (1 kb 5’-gene), gene bodies and 3’UTRs (500 bp 3’-gene) were assessed. *F. graminearum* genes with 5mC sites within genic regions were categorized using FunCat (Functional Catalogue; http://mips.helmholtz-muenchen.de/funcatDB/; Ruepp *et al.* 2004).

3.2.4 Methylation Specific PCR

Methylation Specific PCR (MSP) was used to assess methylation at specific cytosines within the promoter region of *TRI6*. Fungal spores were grown in 24 hour NR, 6 hour NP, and 24 hour NP time points and genomic DNA was isolated using the same conditions used in WGBS experiments. One μg of genomic DNA was subjected to sodium bisulfite conversion using an EpiTect Bisulfite Kit (Qiagen # 59104) as per the manufacturer's instruction. Methylation specific primers were designed using MethPrimer to only anneal to methylated, converted DNA (Li and Dahiya 2002). Non-methylation specific primers were designed to anneal to non-converted DNA as a control for incomplete conversion. Bisulfite converted and non-converted DNA was run with both primer sets individually. Presence or absence, as well as intensity of DNA bands were compared between environmental conditions. Cycle conditions for the 100 ηg PCR reaction: 35 cycles of 52˚C. The primer sequences for MSP can be found in **Supplementary Table T1**.

3.3.1 Analysis of *F. graminearum* DNA methylation by WGBS

Analysis of 5-methylcytosine (5mC) levels in the *F. graminearum* genome was performed through the generation of genome-wide methylation profiles by WGBS in nutrient rich (NR) and nutrient poor (NP) growth media (**Table 1**). Time points were chosen based on gene expression data from RNA sequencing experiments (data not shown). WGBS yielded 2.35 -3.09 million paired end reads, corresponding to a sequencing output of between 0.34 and 0.46 Gb raw data per sample, post quality control. Average read length was between 137.24 and 148.11bp. Conversion rate of bisulfite sequencing was estimated using conversion of the non-methylated Lambda DNA to be 96.5%. Of these sequencing reads, 71.05 to 80.54% of reads were successfully aligned to the *in silico* converted reference genome, resulting in a sequencing depth of 6.52 to 9.25-fold coverage across the 36.5 Mbp genome. As a result of sequencing and alignment, 93 to 97% of the reference genome was covered by at least one sequencing read. A summary of sequencing data can be found in (**Table 1**).

Sequencing was performed with two biological replicates per environmental condition (nutrient poor, NP and nutrient rich, NR), and strong correlation was found between replicates (Pearson Correlation Coefficient = 0.98NP and 0.99NR). A strong correlation was also found between NP and NR conditions (0.97). A dendrogram was produced to group replicates according to their Pearson Correlation Coefficients, by comparing global methylation percentage (**Figure 11**). Replicates from NP conditions grouped most similarly; however NR replicates demonstrated within BR heterogeneity, resulting in the dendrogram in **Figure 11**.

Table 1: Summary of Whole Genome Bisulfite Sequencing Data in *F. graminearum*.

		NR		NP	
Sample	Sequencing Statistics	BR 1	BR 2	BR 1	BR 2
Genome size	36,490,312				
%C+G	48.04				
Gene numbers	11884				
Method	PE[b]				
Conversion rate(%)[a]	96.5				
Read length (bp)		143.3	143.9	146	139.2
Total reads (Mbp)		2,969,861	2,377,556	3,080,927	2,732,924
Raw data(Mb)		768	617	805	698
Number of mapped reads (Mbp)		2,282,961	1,902,698	2,431,564	2,190,676
Mapping efficiency (%)		76.9	80	78.9	80.2
Coverage (%)		93	93	97	93
Mean sequencing depth per strand		7.45x	6.52x	6.72x	9.24x

BR represents biological replicate; NR represents Nutrient Rich conditions, and NP represents Nutrient Poor conditions

[a] Conversion Rate: the conversion rate of BS-seq for *F. graminearum* is calculated from an un-methylated lambda DNA added to the BS-seq library

[b] PE: Paired End Reads

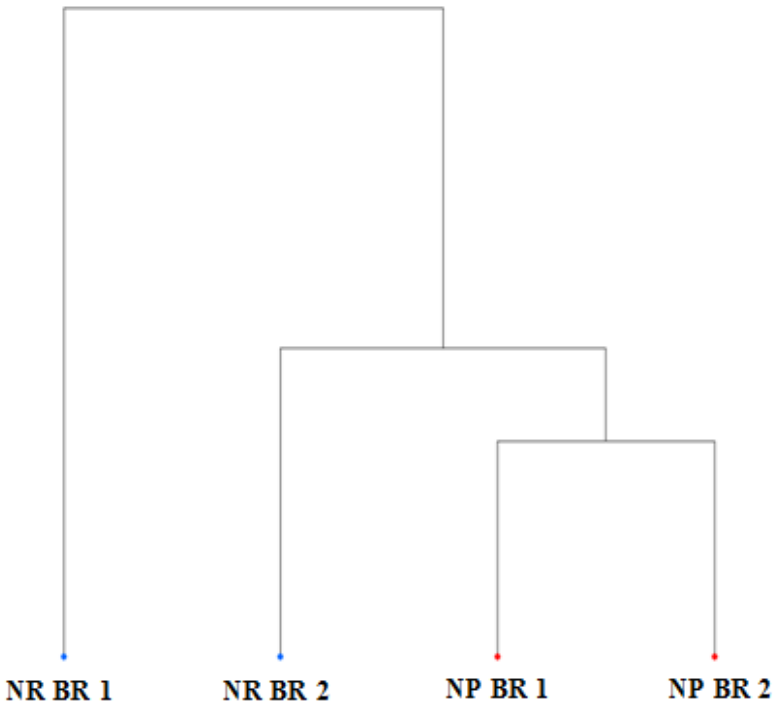


Figure 11: Dendrogram Clustering of WGBS Sequencing Replicates. Clustering of BRs in nutrient rich (NR) and nutrient poor (NP) conditions was assessed according to their Pearson Correlation Coefficient in MethylKit.

3.3.2. Methylation Patterns in the Genome of *F. graminearum*

Genome-wide methylation patterns were assessed by measuring differences in methylation between NR and NP conditions (**Figure 12**). The 10 kb bins with average methylation that is higher in NP conditions are represented below the X-axis (**Figure 12, red**) and bins above the X-axis represent higher average methylation in NR conditions (**Figure 12, blue**). Bins with large methylation differences (>2%) were examined for gene content. The majority of genes were identified as unannotated proteins, although future research may involve characterizing these genes. Gene content per bin is represented at the bottom of each chromosome (**Figure 12, green**). Although, we observe differences in methylation levels between two environmental conditions, no significant differentially methylated regions (DMRs) were detected. It is conceivable that DMRs are present at the gene level; however, software to conduct this high resolution analysis is currently unavailable. Nevertheless, the location of SM clusters and its content are presented (**Figure 12**, Roman numerals; **Table 2**). Interestingly, some of the SM clusters show differential methylation levels between conditions (**Figure 12, Black bars with roman numerals**). As an example, cluster vii on chromosome II is the location of the *TRI* gene cluster.

Figure 12 (next page): DNA methylation patterns are altered under different environmental conditions (nutrient rich, NR; nutrient poor, NP) in *F. graminearum*. The difference between the two conditions as percent methylation is displayed in the primary y-axis in blue for NR condition and red for NP condition. The two conditions (NR and NP) are separated by 0 in the y-axis. The number of genes per 10kb bin is shown in green on the secondary y-axis; secondary metabolite clusters with known products are represented as green bars.

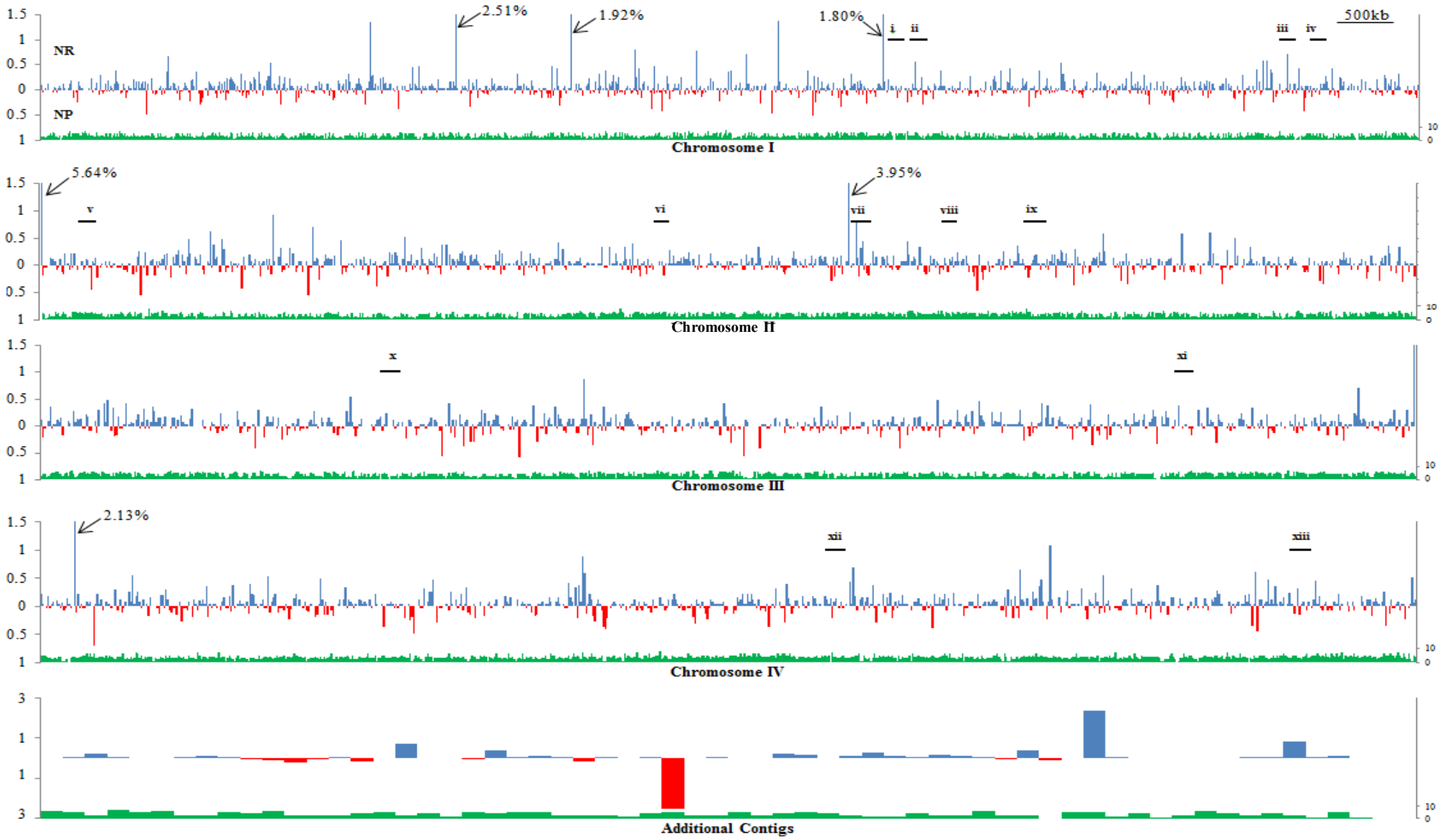

1.5
1
0.5
0
0.5
1
NR
NP
2.51%
1.92%
1.80%
i
ii
iii
iv
500kb
10
0
Chromosome I
5.64%
3.95%
v
vi
vii
viii
ix
Chromosome II
x
xi
Chromosome III
2.13%
xii
xiii
Chromosome IV
3
1
1
3
Additional Contigs

Table 2: Secondary Metabolite Clusters with Known Products in *F. graminearum*.

Location (contig)	Cluster[a]	Genes (number)	Product	Role	Position[b]
Chromosome 1 (63)	C13	*FGSG 02320 - FGSG 02330* (11)	Aurofusarin	Red pigment	i
Chromosome 1 (85)	C15	*FGSG_02393 - FGSG_15983* (9)	Zearalenone	Mycotoxin	ii
Chromosome 1 (34)	C59	*FGSG_10384 - FGSG_10397* (14)	Culmorin	Antifungal, phytotoxic	iii
Chromosome 1 (161)	C60	*FGSG_10458 - FGSG_10464* (7)	Fusarielin	unknown	iv
Chromosome 2 (200)	C49	*FGSG_08077 - FGSG_08084* (8)	Butenolide	Low oral toxicity	v
Chromosome 2 (133)	C28	*FGSG_03064 - FGSG_03067* (4)	Carotenoid	Terpenoid pigment	vi
Chromosome 2 (179)	C23	*FGSG_03529 - FGSG_03543* (13)	Trichothecene	Protein biosynthesis inhibitor	vii
Chromosome 2 (245)	C21	*FGSG_16212 - FGSG_03855* (5)	Triacetylfusarinine	Main extracellular siderophor	viii
Chromosome 2 (134/182)	C18	*FGSG_03956 - FGSG_03971* (17)	Orcinol/orsellinic acid	unknown	ix
Chromosome 3 (253)	C33	*FGSG_16474 - FGSG_05374* (9)	Ferricrocin	Intracellular siderophor	x
Chromosome 3 (10)	C63	*FGSG 11026 - FGSG 11029* (4)	Malonichrome	Extracellular siderophor	xi
Chromosome 4 (97)	C42	*FGSG_07795 - FGSG_16901* (12)	FusarinC	Possible carcinogen / mutagen	xii
Chromosome 4 (156)	C53	*FGSG 09195 - FGSG 17168* (14)	Precursor of perithecial pigment	Black pigment	xii

[a] indicates the cluster designation as defined by Sieber *et al.* (2014)
[b] indicates position of each cluster in **Figure 11**.

Methylation patterns were also examined at the individual cytosine level. We examined the proportions of each cytosine context across all 5mC calls to determine the frequency of each context across the genome. Methylation level of each context was determined by comparing the number of 5mC reads to the total number of reads; this describes the number of times a certain 5mC site is methylated in the fungal population. Under both environmental conditions, the most highly methylated context was CHH, followed by CG and CHG context (**Figure 13**). Methylation in NR conditions occurred in 60% of 5mC cases on a CHH cytosine, 21% of 5mC were on CG cytosine, and 19% of 5mC were on CHG cytosine (**Figure 13A**). Similar results were found in NP conditions (**Figure 13B**). Due to the heterogeneous nature of fungal culture, methylation level (methylated reads / # total reads) was also calculated for each cytosine context, as well as between conditions. On average, cytosine loci displayed a higher methylation level in NP conditions (Figure 3C). Similar to other fungi, we observed that cytosine in the CHH context displayed the highest methylation level at 8.38% and 11.13% in NR and NP conditions, respectively (**Figure 13C**). Methylation levels of CHG cytosine were lowest in CHG contexts, with 5.70% and 6.48% in NR and NP conditions, respectively and methylation levels of CG context cytosine were 6.36% and 7.15%, respectively (**Figure 13C**).

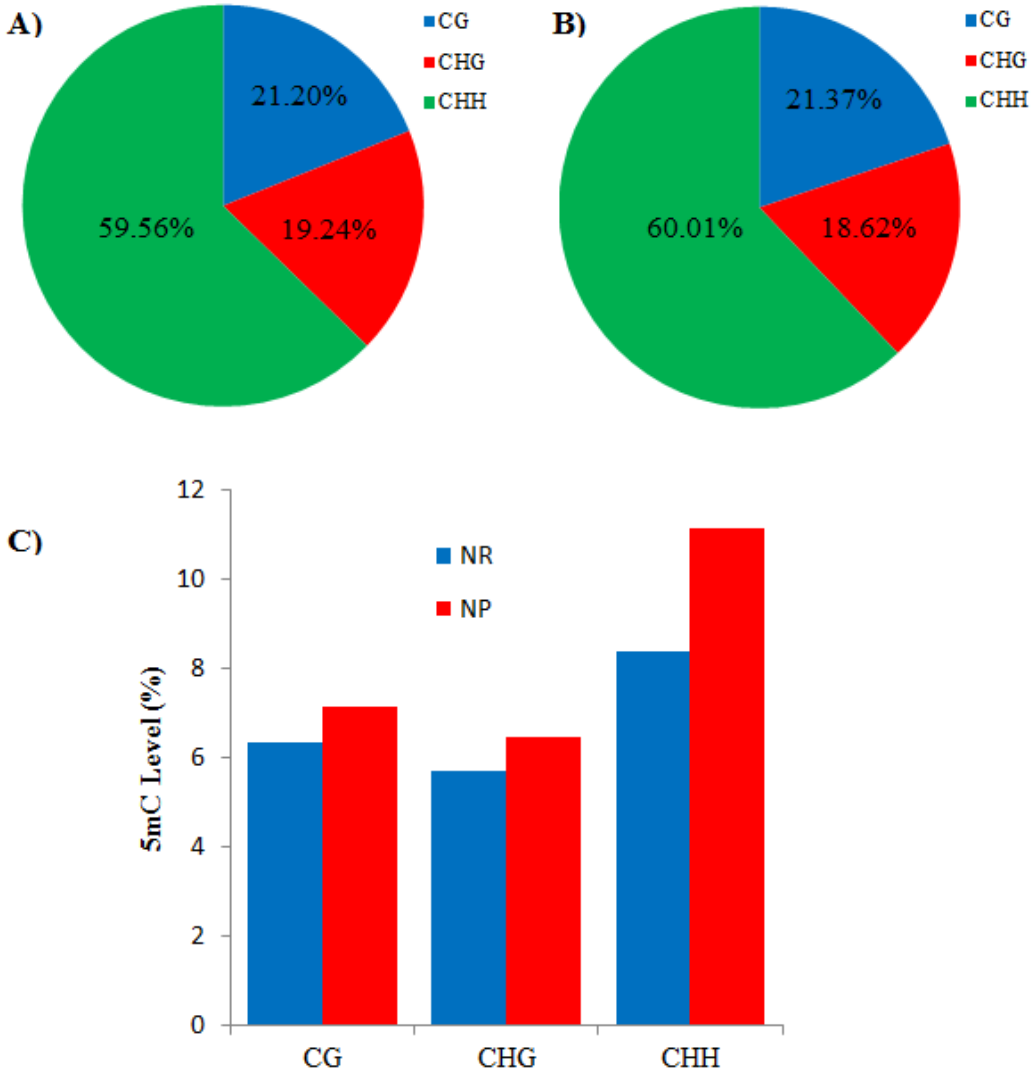


Figure 13: Methylation in *F. graminearum* is primarily found on CHH cytosine. A) Percent occurrence of 5mC contexts across two replicates of nutrient rich growth (NR). B) Percent occurrence of 5mC contexts across two replicates of nutrient poor condition growth (NP). C) The average population methylation level (# 5mC / #C) at each 5mC loci across three cytosine contexts,

Next, the number of 5mC sites that passed the binomial correction was used to determine the average methylation percent genome wide (# 5mC / Total # C) (**Figure 14**). Of the 8,764,650 cytosine bases in the *F. graminearum* genome, 9,660 sites in biological replicate (BR) 1 and 8,022 sites in BR 2 were 5mC sites, resulting in an average genome methylation of 0.110% and 0.092%, respectively for each biological replicate (**Figure 14A**). This represents an overall average of methyl cytosines of 0.1% in *F. graminearum* in NR conditions. As indicated in the Venn diagram, we observed a total of 3,430 sites that are conserved between the two replicates. Given the heterogeneity of the fungal culture in the NR conditions, these conserved sites might indicate important regulatory marks for the particular environmental condition. In NP conditions, 3,491 and 4,931 sites were observed in BR 1 and BR 2, resulting in an average methylation of 0.070% and 0.086%, respectively (**Figure 14B**). This represents an average genome methylation of 0.078% in NP conditions. A total of 2,588 conserved sites were observed within NP conditions (**Figure 14B**). A comparison between conserved sites between NR (3,430) and NP (2,588) revealed that 1,439 sites and 597 sites are unique to NR and NP conditions, respectively, and a total of 1,991 methylation sites that are common to all environmental conditions (**Figure 14C**).

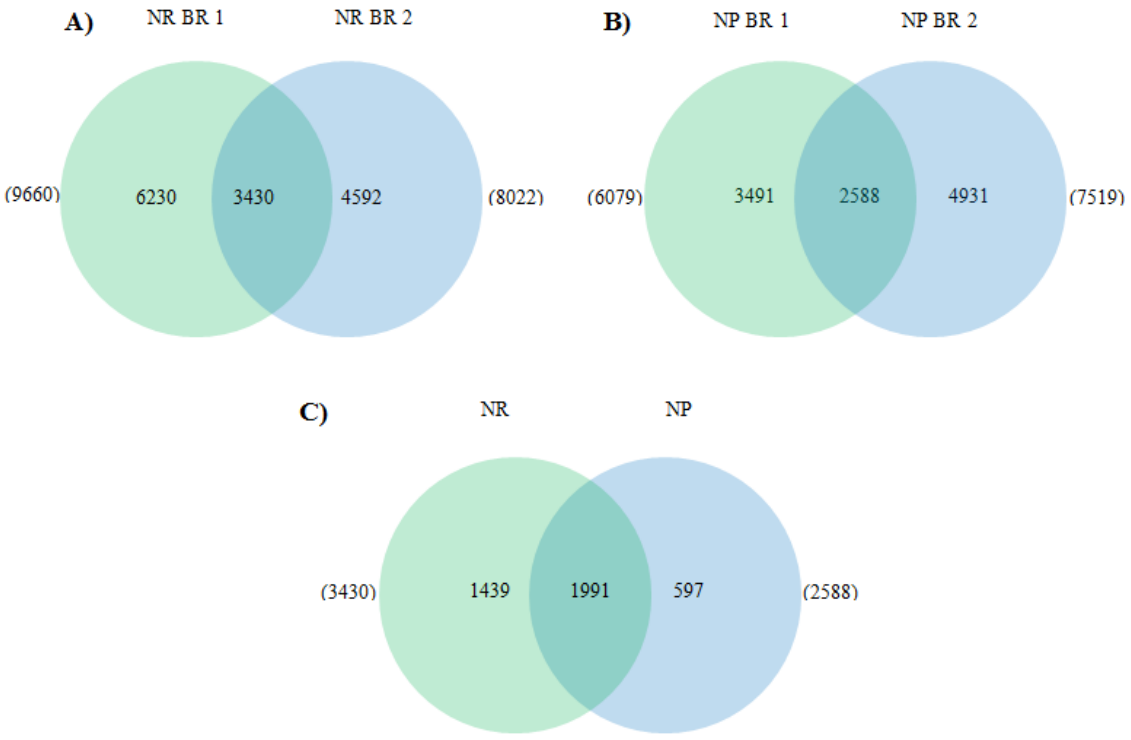


Figure 14: Highly conserved 5-methylcytosine sites (5mC) are present in the genome of *F. graminearum*. A) Conserved 5mC sites between replicates in nutrient rich (NR) conditions. B) Conserved 5mC sites between replicates in nutrient poor (NP) conditions. C) Highly conserved 5mC sites found in all replicates and conditions.

We examined the 1,439 5mC sites that were unique from NR conditions (**Figure 14C**). These sites were identified by their position in intergenic and genic regions (1 kb promoter, gene region and 500 bp 3' UTR). The majority of sites were identified in intergenic regions. We observed 139 positions in genic regions corresponding to 33 genes (**Table 3**). FunCat analysis (Functional Catalogue; http://mips.helmholtz-muenchen.de/funcatDB/; Ruepp *et al.* 2004) was performed on these genes and the resulting classifications are in **Table 5**. Enriched genes are defined as genes which occur at a higher frequency in our subset than would be expected given the proportion of these genes in the genome (significantly enriched genes are supported by a *P*-value < 0.05). Interestingly, we observed a number of genes enriched in our sample involved in primary metabolism and growth, including amino acid biosynthesis (*FGSG_17683*), nucleotide metabolism *(FGSG_05668*), steroid biosynthesis (*FGSG_01959*, *FGSG_08187*), and cell wall development (*FGSG_04752*). Genes involved in environmental sensing and interaction (*FGSG_02449*, *FGSG_04752*) are also enriched (**Table 4**).

Table 3: Localization of unique 5mC sites in genic regions of *F. graminearum* under nutrient rich conditions.

Condition	# Methylation Sites	Gene	Genic Feature	Annotation
NR	1	*FGSG_00946*	Gene	related to heat shock transcription factor
	6	*FGSG_01335*	Gene	conserved hypothetical protein
	4	*FGSG_01959*	3' UTR	probable cytochrome P450 (involved in C-22 denaturation of the ergosterol side-chain)
	1	*FGSG_02449*	Gene	related to nik-1 protein (Os-1p protein)
	5	*FGSG_03518*	Gene	conserved hypothetical protein
	2	*FGSG_04474*	Gene	related to SRP40 - suppressor of mutant AC40 of RNA polymerase I and III
	1	*FGSG_04752*	Promoter	related to chitinase
	3	*FGSG_04824*	Gene	conserved hypothetical protein
	2	*FGSG_05334*	Gene	conserved hypothetical protein
	5	*FGSG_05701*	Gene	related to quinic acid utilisation protein QUTG (inositol-1(or 4)-monophosphatase)
	4	*FGSG_06385*	Gene	pathogenicity MAP kinase 1
	2	*FGSG_08187*	3' UTR	related to cytochrome P450 monooxygenase (lovA)
	3	*FGSG_09106*	Gene	conserved hypothetical protein
	3	*FGSG_09576*	Gene	conserved hypothetical protein
	4	*FGSG_10385*	Gene	probable centractin (ro-4)
	7	*FGSG_12281*	Gene	related to cyclin B1 interacting protein
	7	*FGSG_12367*	3' UTR	conserved hypothetical protein
	2	*FGSG_12935*	Promoter	conserved hypothetical protein
	2	*FGSG_13109*	Gene	conserved hypothetical protein
	1	*FGSG_13483*	Gene	related to n-carbamoyl-l-amino acid hydrolase
	3	*FGSG_13616*	Promoter	probable thioredoxin
	1	*FGSG_14020*	Gene	conserved hypothetical protein
	5	*FGSG_15367*	3' UTR	hypothetical protein
	6	*FGSG_16151*	Promoter	hypothetical protein
	2	*FGSG_16726*	Promoter	related to TVP38 - Integral membrane protein localized to vesicles along with the v-SNARE Tlg2p
	1	*FGSG_17397*	Promoter	hypothetical protein
	2	*FGSG_17683*	Gene	related to anthranilate synthase component
	13	*FGSG_02284*	3' UTR	conserved hypothetical protein
	3	*FGSG_05668*	Promoter	probable galactose-1-phosphate uridylyltransferase
	2	*FGSG_10083*	Gene	conserved hypothetical protein
	2	*FGSG_15156*	Gene	conserved hypothetical protein
	3	*FGSG_01725*	Promoter	related to heterokaryon incompatibility protein
	31	*FGSG_01146*	3' UTR	conserved hypothetical protein

Legend: NP, Nutrient Poor Conditions; Promoter, 1kb upstream of ATG; 3' UTR, 500bp 3' of gene.

Table 4: Functional categorization of genes with 5mC sites unique to nutrient rich conditions.

FUNCTIONAL CATEGORY	Genes	*P*-VALUE
01 METABOLISM		
01.01.09.06.01 biosynthesis of tryptophan	*FGSG_17683*	0.05
01.05.02.01 nucleotide-sugar metabolism	*FGSG_05668*	0.04
01.05.03.03.07 chitin catabolism	*FGSG_04752*	0.03
01.06.06 isoprenoid metabolism	*FGSG_01959 FGSG_05334 FGSG_08187*	0.01
01.06.06.07 diterpenes metabolism	*FGSG_05334 FGSG_08187*	0.00
01.06.06.11 tetracyclic and pentacyclic triterpenes (cholesterin, steroids and hopanoids) metabolism	*FGSG_01959 FGSG_08187*	0.04
16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT		
16.21.01 heme binding	*FGSG_01959 FGSG_05334 FGSG_08187*	0.00
16.25 oxygen binding	*FGSG_01959*	0.02
20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES		
20.01.15 electron transport	*FGSG_01959 FGSG_05334 FGSG_08187 FGSG_13616*	0.01
20.09.14.02 actin dependent transport	*FGSG_10385*	0.03
30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM		
30.05.01.10 two-component signal transduction system (sensor kinase component)	*FGSG_02449*	0.04
32 CELL RESCUE, DEFENSE AND VIRULENCE		
32.05.03 defense related proteins	*FGSG_05334 FGSG_08187*	0.02
32.07 detoxification	*FGSG_01959 FGSG_02449 FGSG_05334 FGSG_08187 FGSG_13616*	0.01
32.07.01 detoxification involving cytochrome P450	*FGSG_01959 FGSG_05334 FGSG_08187*	0.00
32.07.07 oxygen and radical detoxification	*FGSG_02449 FGSG_13616*	0.01
34 INTERACTION WITH THE ENVIRONMENT		
34.11.03.11 pH response	*FGSG_04752*	0.05
34.11.10 response to biotic stimulus	*FGSG_04752*	0.04
36 SYSTEMIC INTERACTION WITH THE ENVIRONMENT	*FGSG_02449*	0.04
36.20 plant / fungal specific systemic sensing and response	*FGSG_02449*	0.04
36.20.18 plant hormonal regulation	*FGSG_02449*	0.04

Enriched categories of genes are significant at $\alpha < 0.05$.

Of the 597 sites that were conserved in only NP conditions, 102 sites were found within genic regions, corresponding to 33 *F. graminearum* genes (**Table 5**). FunCat analysis of these genes indicated an enrichment of threonine degradation (*FGSG_04703*), metabolism of amino acids (*FGSG_05668*), and peptide transport (*FGSG_03279*; **Table 6**).

We also identified 1,991 conserved 5mC sites found in all BRs in both nutrient conditions (**Figure 14C**). Of these, 202 localised to genic region corresponding to 32 genes (**Table 7**). These sites were primarily found within the coding sequence, although a number of sites were also found in the promoter and UTR regions. FunCat analysis showed enrichment genes in primary metabolism, cell rescue, defence and virulence, development, and cell fate, as well as interaction with the environment (**Table 8**). These results suggest a strong enrichment of genes related to the environment. In addition, we also observed genes enriched in chromatin remodelling (*FGSG_09218)* and in transcription repression (*FGSG_00946* and *FGSG_10325*).

Table 5: Localization of unique 5mC sites in genic regions of *F. graminearum* under nutrient poor conditions.

Condition	# Methylation Sites	Gene	Genic Feature	Annotation
NP	10	*FGSG_00913*	Gene	conserved hypothetical protein
	1	*FGSG_02035*	Gene	related to vegetatible incompatibility protein HET-E-1
	1	*FGSG_02047*	Gene	conserved hypothetical protein
	2	*FGSG_02066*	3' UTR	related to HXT2 - Hexose facilitator of moderately low affinity for glucose
	2	*FGSG_02067*	Promoter	related to raffinose invertase
	2	*FGSG_02322*	Gene	aurofusarin/rubrofusarin efflux pump AFLT
	3	*FGSG_02983*	Gene	conserved hypothetical protein
	1	*FGSG_03278*	3' UTR	related to peroxisomal amine oxidase (copper-containing)
	1	*FGSG_03279*	Promoter	related to sexual differentiation process protein isp4
	11	*FGSG_03321*	Gene	conserved hypothetical protein
	1	*FGSG_03483*	Gene	probable pectin lyase precursor
	3	*FGSG_03483*	Promoter	probable pectin lyase precursor
	1	*FGSG_03682*	3' UTR	conserved hypothetical protein
	5	*FGSG_04228*	3' UTR	hypothetical protein
	6	*FGSG_04582*	Gene	probable glutamate--tRNA ligase
	2	*FGSG_04703*	Promoter	related to formaldehyde dehydrogenase
	3	*FGSG_05640*	Promoter	probable ribosomal protein L17.e, cytosolic
	2	*FGSG_08695*	3' UTR	conserved hypothetical protein
	9	*FGSG_09004*	Gene	probable phosphatidylglycerophosphate synthase PEL1
	1	*FGSG_09005*	3' UTR	related to nadh-ubiquinone oxidoreductase subunit b17.2
	5	*FGSG_10008*	3' UTR	conserved hypothetical protein
	1	*FGSG_10325*	Gene	conserved hypothetical protein
	5	*FGSG_10992*	Gene	related to polysaccharide deacetylase
	6	*FGSG_12113*	Gene	conserved hypothetical protein
	3	*FGSG_12532*	Gene	conserved hypothetical protein
	5	*FGSG_15234*	Promoter	hypothetical protein
	2	*FGSG_16722*	Gene	related to MUTS protein homolog 1
	1	*FGSG_02284*	3' UTR	conserved hypothetical protein
	2	*FGSG_05668*	Promoter	probable galactose-1-phosphate uridylyltransferase
	1	*FGSG_10083*	Gene	conserved hypothetical protein
	1	*FGSG_15156*	Gene	conserved hypothetical protein
	1	*FGSG_01725*	Promoter	related to heterokaryon incompatibility protein
	2	*FGSG_01146*	3' UTR	conserved hypothetical protein

Legend: NP, Nutrient Poor Conditions; Promoter, 1kb upstream of ATG; 3' UTR, 500bp 3' of gene.

Table 6: Functional categorization of genes with 5mC sites unique to nutrient poor conditions.

FUNCTIONAL CATEGORY	Genes	*P*-VALUE
01 METABOLISM		
01.01.06.04 metabolism of threonine	*FGSG_04703*	0.04
01.01.06.04.02 degradation of threonine	*FGSG_04703*	0.03
01.05.02.01 nucleotide-sugar metabolism	*FGSG_05668*	0.04
20 CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES		
20.01.09 peptide transport	*FGSG_03279*	0.05

Enriched categories of genes are significant at $\alpha < 0.05$.

Table 7: Localization of common 5mC sites in genic regions of *F. graminearum* in both nutrient rich and nutrient poor conditions.

Condition	# Shared Methylation Sites	Gene	Genic Feature	Annotation
NR & NP	4	*FGSG_00448*	Gene	probable pre-mRNA splicing factor ATP-dependent RNA helicase PRP16
	6	*FGSG_00946*	Gene	related to heat shock transcription factor
	8	*FGSG_02035*	Gene	related to vegetative incompatibility protein HET-E-1
	12	*FGSG_02066*	3' UTR	related to HXT2 - Hexose facilitator of moderately low affinity for glucose
	2	*FGSG_02322*	Gene	aurofusarin/rubrofusarin efflux pump AFLT
	7	*FGSG_02449*	Gene	related to nik-1 protein (Os-1p protein)
	9	*FGSG_03081*	Gene	conserved hypothetical protein
	4	*FGSG_04703*	Promoter	related to formaldehyde dehydrogenase
	6	*FGSG_04752*	Promoter	related to chitinase
	7	*FGSG_04841*	Gene	conserved hypothetical protein
	7	*FGSG_05216*	Gene	probable cytokinesis protein SepA
	9	*FGSG_05334*	Gene	conserved hypothetical protein
	12	*FGSG_05633*	Promoter	conserved hypothetical protein
	3	*FGSG_05845*	Gene	probable protein kinase 1
	5	*FGSG_06575*	3' UTR	conserved hypothetical protein
	6	*FGSG_09218*	Gene	related to Hst1p and Sir2p
	8	*FGSG_09471*	Gene	probable glucose-regulated protein 78 of hsp70 family
	1	*FGSG_10083*	Gene	conserved hypothetical protein
	4	*FGSG_10325*	Gene	conserved hypothetical protein
	1	*FGSG_11746*	3' UTR	hypothetical protein
	10	*FGSG_11978*	Gene	conserved hypothetical protein
	6	*FGSG_12196*	Gene	hypothetical protein
	2	*FGSG_12935*	Promoter	conserved hypothetical protein
	3	*FGSG_13109*	Gene	conserved hypothetical protein
	9	*FGSG_13743*	Promoter	probable beta transducin-like protein
	1	*FGSG_14020*	Gene	hypothetical protein
	1	*FGSG_15156*	Gene	hypothetical protein
	12	*FGSG_15171*	Promoter	hypothetical protein
	5	*FGSG_16363*	3' UTR	hypothetical protein
	17	*FGSG_17269*	3' UTR	hypothetical protein
	13	*FGSG_17343*	Gene	hypothetical protein
	2	*FGSG_17683*	Gene	related to anthranilate synthase component

Legend: NP, Nutrient Poor Conditions; Promoter, 1kb upstream of ATG; 3' UTR, 500bp 3' of gene.

Table 8: Functional categorization of genes with common 5mC sites in *F. graminearum* in both NR and NP conditions.

FUNCTIONAL CATEGORY	Genes	*P*-VALUE
01 METABOLISM		
01.01.06.04 metabolism of threonine	*FGSG_04703*	0.04
01.01.06.04.02 degradation of threonine	*FGSG_04703*	0.03
01.01.09.06.01 biosynthesis of tryptophan	*FGSG_17683*	0.05
01.04 phosphate metabolism	*FGSG_05216 FGSG_05845 FGSG_09218 FGSG_09471*	0.05
01.05.03.03.07 chitin catabolism	*FGSG_04752*	0.03
01.06.06.07 diterpenes metabolism	*FGSG_05334*	0.03
10 CELL CYCLE AND DNA PROCESSING		
10.03 cell cycle	*FGSG_00946 FGSG_05216 FGSG_05845 FGSG_09218 FGSG_09471*	0.02
10.03.01 mitotic cell cycle and cell cycle control	*FGSG_00946 FGSG_05845 FGSG_09218 FGSG_09471*	0.03
10.03.01.01.13 M/G1 transition of mitotic cell cycle	*FGSG_09218*	0.01
11 TRANSCRIPTION		
11.02.03.04.03 transcription repression	*FGSG_00946 FGSG_09218 FGSG_10325*	0.00
14 PROTEIN FATE (folding, modification, destination)		
14.01 protein folding and stabilization	*FGSG_00946 FGSG_09471*	0.04
18 REGULATION OF METABOLISM AND PROTEIN FUNCTION		
18.01.07 regulation by binding / dissociation	*FGSG_00946 FGSG_09218*	0.03
***20* CELLULAR *T*RANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES**		
20.09.05 non-vesicular ER transport	*FGSG_09471*	0.03
30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM		
30.01 cellular signalling	*FGSG_02449 FGSG_05216 FGSG_05845 FGSG_13743*	0.04
30.01.09.07 cAMP/cGMP mediated signal transduction	*FGSG_05845*	0.04
30.05.01.10 two-component signal transduction system (sensor kinase component)	*FGSG_02449*	0.04
32 CELL RESCUE, DEFENSE AND VIRULENCE		
32.01 stress response	*FGSG_00946 FGSG_02449 FGSG_05216 FGSG_05845 FGSG_09218 FGSG_09471*	0.00
32.01.01 oxidative stress response	*FGSG_05845 FGSG_09218*	0.03
32.01.03 osmotic and salt stress response	*FGSG_02449 FGSG_05216*	0.02
32.01.05 heat shock response	*FGSG_00946 FGSG_09471*	0.00
32.01.07 unfolded protein response (e.g. ER quality control)	*FGSG_00946 FGSG_09471*	0.01
34 INTERACTION WITH THE ENVIRONMENT		
34.11 cellular sensing and response to external stimulus	*FGSG_00946 FGSG_02066 FGSG_02449 FGSG_04752 FGSG_05845*	0.00
34.11.03.11 pH response	*FGSG_04752*	0.05
34.11.09 temperature perception and response	*FGSG_00946*	0.05
34.11.10 response to biotic stimulus	*FGSG_04752*	0.04
34.11.12 perception of nutrients and nutritional adaptation	*FGSG_02066 FGSG_04752 FGSG_05845*	0.00
36 SYSTEMIC INTERACTION WITH THE ENVIRONMENT		
36.20 plant / fungal specific systemic sensing and response	*FGSG_02449*	0.04
36.20.18 plant hormonal regulation	*FGSG_02449*	0.04
40 CELL FATE	*FGSG_02449 FGSG_05216 FGSG_05845 FGSG_09218*	0.04
41 DEVELOPMENT (Systemic)		
41.01 fungal/microorganismic development	*FGSG_09471 FGSG_13743*	0.02
42 BIOGENESIS OF CELLULAR COMPONENTS		
42.10 nucleus	*FGSG_00946 FGSG_09218 FGSG_09471*	0.03

Enriched categories of genes are significant at $\alpha < 0.05$.

3.3.3. 5mC dynamics in *TRI6* may Reflect Phenotypic Differences in 15-ADON Biosynthesis

In Chapter 2, we observed significant differences in 15-ADON levels in methyltransferase double deletion mutant strain *dnmt1/dnmt4* (**Figure 6**). We were interested to know if there was a link between the production of 15-ADON and methylation patterns in the promoter region of the regulatory gene *TRI6*. WGBS identified nine 5mC sites in *TRI6*, and we chose 4/9 positions randomly in the promoter region of *TRI6* for methylation specific PCR (MSP) analysis in three different environmental conditions: 24 hour nutrient rich (NR), 6 hour nutrient poor (NP), and 24 hour NP (**Figure 15**). Since the PCR band intensity is directly proportional to methylation level, we determined the level of methylation qualitatively by band intensity at these methylation sites in the promoter of *TRI6*. The analysis showed that for position -426, there were no significant differences between *WT* and the double mutant. However, there was a difference in the methylation at 6 hours in NP for both the strains compared to either 24 hours NR or 24 hours NP conditions (**Figure 15A**). This position served as a positive control for the analysis of other positions in the *TRI6* promoter, as the same amount of DNA was used in all the experiments. Analysis of methylation site -350 showed increased methylation at 6 hours NP compared to 24 hour NR for *WT* and the double mutant, however, at 24 hours NP, the double mutant had a dramatic decrease in methylation compared to *WT* (**Figure 15B**). A similar pattern was observed for the position -262/260, but additionally, we also observed differences at 24 hours NP conditions (**Figure 15C**). It should be noted that conversion rate of the bisulfite treatment was assessed using the same converted DNA as template with non-methylation specific

primers. Any amplification would indicate incomplete conversion resulting in primer binding. We observed no amplification in these samples indicating complete conversion (data not shown). Overall, MSP results show strong correlation with the changes in methylation and the accumulation of mycotoxin levels. Specifically, a decrease in methylation of positions -350 and -262/-260 in the double mutant corresponded to increase levels of 15-ADON accumulation.

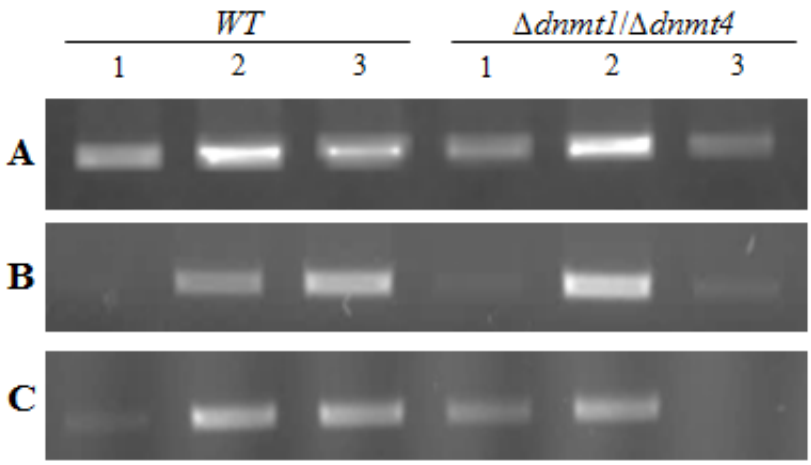


Figure 15: Methylation specific PCR of DNA methylation sites within the promoter regions of *TRI*6. Methylation levels were assessed for *WT* and Δ*dnmt1*/Δ*dnmt4* strains at four cytosine loci (A: -426; B: -350; C: -262/-260) across three environmental conditions (1: 24h NR; 2: 6h NP; 3: 24h NP). Primers for MSP can be found in **Supplemental Table T1**. This is representative of two independent experiments.

Chapter 3 - Discussion

Epigenetic processes are those that can alter phenotype without an underlying change in genotype (Handy *et al*. 2011). The processes work together to contribute to gene expression and genome stability, and may serve as a link between the genome and the environment. One such epigenetic mechanism is DNA methylation. Methylation of cytosine bases (5mC) has already been implicated in a number of important cellular processes, from X-chromosome inactivation and tumorigenesis in mammals, transposon silencing in plants, and genome defence in fungi (Sharp *et al.* 2011; Robertson 2005; Lippman *et al.* 2004; Rountree and Selker 2010).

Methylation levels in fungi vary, ranging from barely detectable levels to 3% in some species (Zhu *et al.* 2015; Kouzminova and Selker 2001; Jeon *et al.* 2015; Capuano *et al.* 2014). DNA methylation has been investigated primarily for its role in silencing repetitive elements and transposable elements in fungi, however, evidence that DNA methylation in fungi can regulate transcriptional activity leading to phenotypic changes exists (Jeon *et al.* 2015). Moreover, even at the low levels, transcriptional changes correlated to DNA methylation has been observed in *C. albicans* (Mishra *et al.* 2011).

In *F. graminearum*, we performed WGBS between two environmental conditions, nutrient rich (NR) and nutrient poor (NP). Methylation levels were found to be comparable to related fungi; DNA methylation in *M. oryzae* identified methylation percentages at ~0.22%, *C. albicans* DNA methylation percentage varied between 0.05-0.1% depending on the developmental stage, and *Cordyceps militaris* with 0.4% (Jeon *et al.* 2015; Mishra *et al*. 2011; Wang *et al.* 2015).

A low level of methylation and heterogeneity between biological replicates poses a unique challenge to assigning DMRs (Ziller *et al.* 2015). Methylation coverage recommendations suggest that read depths of 5-15x are satisfactory for sensitivity and specificity for WGBS analysis, however, we were unable to define statistically significant DMRs (regions of 1 kb with significantly different levels of 5mC, compared between conditions by Fisher's Exact Test; Ziller *et al.* 2015). Using MethylKit we observed clustering of NP replicates, while some heterogeneity was observed between NR samples based on the Pearson Correlation Coefficient (**Figure 11**), reiterating the suggestion that methylation is a dynamic process, adapting to changing environmental conditions.

To better understand heterogeneity within conditions, we also examined DNA methylation contexts and levels of cytosine residues across the genome (**Figure 13**). Methylation context can provide information on the role and mechanism of DNA methylation in *F. graminearum*. For example, methylation occurring in the symmetrical contexts (CG, CHG) are typically propagated by maintenance methyltransferases that preferentially recognize and methylate hemi-methylated sites, such as those found on newly replicated DNA (Kouzminova and Selker 2001). Asymmetrical sites may be the product of *de novo* methylation (Law and Jacobsen 2010). Thus, presence of high levels of CHH methylation present in *F. graminearum* suggests that a *de novo* methyltransferase activity may be dominant in this fungus. In a related fungi *M. oryzae,* a preference for CHH and CG methylation was observed (Jeon *et al.* 2015).

In addition to differences between samples, heterogeneity is also observed within a population. To lend confidence to methylation sites assigned by our analyses, we examined 5mC sites in both replicates and conditions (**Figure 14**). This technique was

employed by Xiang *et al.* (2010) to provide a very conservative estimate of true 5mC sites in the genome of the *Dazao* silkworm. When this technique was applied in *Aspergillus flavus,* no conserved sites were observed between replicates, leading the authors to conclude that DNA methylation is absent in this fungus (Liu *et al.* 2012). Our analysis of the *F. graminearum* genome confidently estimated ~3,400 5mC sites that overlapped between NR replicates and ~2,500 sites that overlapped between NP replicates (**Figures 14A and 14B**). These sites were considered conserved sites in each condition, and provided strong evidence that DNA methylation is present in the *F. graminearum* genome.

In addition to the conserved sites, we also identified methylation sites that were unique to each environmental condition (**Figure 14C**). Of the 1,439 sites present only in NR conditions, 139 sites corresponded to 33 genes (**Table 3**). The function of these 5mC marks remains unclear. It is possible that DNA methylation can act both as a repressive and an activation mark for transcription factors or methyl-binding proteins to recruit transcription machinery. More research is required to understand the significance of these methylation sites that are found conserved in specific environmental conditions.

Of the 597 5mC unique sites identified only in NP conditions, 102 of these sites were found in genic regions and similar to NR conditions, these were primarily within the coding sequence (**Table 5**). To date, no studies have compared the effects of DNA methylation in the coding sequences *versus* the methylation in the promoter regions. Additional research is required to identify the significance of DNA methylation found in different genic regions. Functional categorization of the 33 genes with 5mC sites identified only three enriched categories: amino acid degradation, nucleotide metabolism,

and peptide transport (**Table 6**). Under nutrient deprived conditions, it remains plausible that degradation of amino acids such as threonine is a response to stress to remobilize nutrients to adapt to the new environment.

We also examined potential roles for the 1,991 5mC sites, conserved in both environmental conditions by identifying the number of sites that reside in genic regions. We found a total of 202 sites present in the genic region of 32 genes, with the majority of the sites localized within the coding sequence (**Table 7**). We observed that genes with highly conserved 5mC mark were involved in transcriptional repression (*FGSG_00946, FGSG_09218* and *FGSG_10325*). For example *FGSG_00946* encodes a heat shock transcription factor, potentially involved with chromatin structure, in response to stress. *FGSG_09218* is annotated as relating to Hst1p and Sir2p, NAD-dependent histone deactylases, capable of acting as transcriptional repressors through modification of chromatin structure. Finally, *FGSG_10325* is a conserved hypothetical protein, but is linked to biological process involved in nitrogen metabolism through transcriptional repression (**Table 8**). We also identified a number of genes related to signalling responses; *FGSG_05845* is involved in cAMP/cGMP mediated signal transduction in the perception of nutrients and nutritional adaptation. Although, the function of these methylation marks remains a mystery, nevertheless, these marks appeared consistently in nutrient related and transcription related genes. This suggests a role for DNA methylation in regulating gene expression as result of changing environmental conditions. An analysis of the *F. graminearum* methylome under various conditions and stresses may provide further clues to this process. As well, MSP can be employed to establish a link between specific 5mC sites under various stress conditions.

Although methylation was low across the genome, we observed differences in DNA methylation between environmental conditions that were above the genome average (**Figure 12**). Interestingly, the majority of bins contained higher average methylation in NR conditions (blue, **Figure 12**), suggesting a possible role for DNA methylation in repression of genes not required during nutrient rich growth. For example, genes involved in utilization of non-preferred nutrient sources, or even expression of secondary metabolites may be repressed by greater methylation (Mishra *et al.* 2011). Indeed, average methylation is variable in areas of SM gene clusters (Sieber *et al.* 2014). An example here included the *TRI* gene cluster (cluster vii, **Figure 12**), displaying large differences in methylation between nutrient conditions. These results indicate that methylation differences occur between conditions, even in SM clusters (**Figure 12** & **Table 3**).

A notable phenotype observed in the double deletion of *DNMT*s in Chapter 2 was an increase in 15-ADON production. Since 15-ADON biosynthesis is regulated by *TRI6*, we used MSP to assess methylation dynamics in the promoter regions of *TRI6* under various environmental conditions (**Figure 15)**. Unlike WGBS, MSP is a high-resolution technique to map individual methylation sites. The results confirmed that promoter region methylation sites of *TRI6* are regulated by DMTs and environmental conditions. One of the unexpected observations was that environmental conditions affected methylation patterns in the double deletion strain (**Figure 15B**). These results suggest a presence of additional and as of yet, undescribed DMTs in *F. graminearum*. A preliminary result using high resolution liquid chromatography mass spectroscopy (LC-MS) supports our genetic results and indicated residual DNA methylation activity in the double mutant

strain (personal communication, Nadia Ponts). Homology and domain searches discovered no additional annotated DMTs, however, it is possible that residual DNA methylation observed in the double mutant strain is due to the action of yet unknown mechanisms.

In this study, we observed genome-wide methylation in *F. graminearum* of ~0.1% by WGBS. In addition to establishing the presence of DNA methylation, differences in methylation percent was also observed between environmental conditions, suggesting a link between DNA methylation and the environment. We discovered unique 5mC sites exist within genic regions of genes involved in important cellular processes such as signalling and nutrient utilization, as well as virulence and SM production. Establishing the presence of DNA methylation is only the first step in understanding the role this epigenetic process plays in *F. graminearum*. Future research is necessary to elucidate the relationship between epigenetic marks and gene expression

Chapter 4: Epigenetics of a Cereal Killer

Chapter 4 - Discussion

F. graminearum is a fungal phytopathogen with a broad host range among economically important small grain cereal crops. Understanding regulatory mechanisms in the pathogen responsible for virulence is critical to combatting the disease. We undertook a whole genome methylome approach to study DNA methylation in *F. graminearum* with the objective to determine a role for DNA methylation in both fungal development and pathogenicity.

We identified two putative DNA methyltransferase genes in *F. graminearum*, *DNMT1* and *DNMT4*, and through targeted gene deletions examined potential roles of the two *Fg*DMTs in both development and pathogenicity. Our results demonstrated that each *Fg*DMTs have a crucial role in developmental processes, such as growth and sexual spore development, and pathogenicity related processes, such as secondary metabolism production and virulence. We also observed that phenotype severity increased when both the genes were deleted, suggesting the DMTs act synergistically. The genetic evidence was supported by WGBS that demonstrated that DNA methylation is present in *F. graminearum* genome, at levels consistent with those observed in related fungi.

DNA methylation in *F. graminearum* appears to be dynamic and able to respond to changes in environment. These epigenetic changes may then result in downstream phenotypes, possibly influencing both development and pathogenicity or secondary metabolism related pathways through yet unknown mechanisms. For example, we observed 5mC sites conserved in nutrient rich conditions within the genic region of

growth and development, transport, signalling and environmental interaction related genes. An aberrant methylation pattern on these genes may contribute to the development phenotypes observed in *DNMT* mutants, such as differences in vegetative growth. We also observed genes with conserved 5mC sites in nutrient poor conditions. For instance, genes involved in threonine degradation process are linked to leucine metabolism. Previous studies from our lab have implicated leucine metabolism in the regulation of *TRI6* expression and DON production in *F. graminearum* (Subramaniam *et al*. 2015). De-regulation of this process by disruption in DMTs may be in part responsible for the observed phenotype of increased DON production in the double deletion strain. Conserved 5mC sites were also observed in the promoter region of several sexual differentiation process genes and it is plausible that similar de-regulation of this type of epigenetic modification may be partly responsible for the ascospore phenotype. Interestingly, we also observed sites conserved within all conditions (highly conserved sites). Some of these sites were found in genes encoding for transcriptional repressors, such as chromatin structure modifying proteins. De-regulation of these transcriptional modifiers may have an even larger impact on fungal development. Together, these results suggest a potential role for DNA methylation in regulating important genetic processes such as development and pathogenicity.

Given the overall low methylation levels found in fungi, bisulfite conversion in the WGBS process can have a major impact on assigning methylation sites. Incomplete conversion of the genomic DNA can result in a non-methylated cytosine assigned as 5mC during subsequent mapping and alignment, giving rise to false positives (Kurdyukov and Bullock 2016). On the other hand, stringency associated with binomial model statistics

will likely have resulted in omission of other potential 5mC sites in *F. graminearum* genome. These sites may contribute to the regulation of other developmental or pathology related processes. Identifying such sites is one possible future direction of this study.

Novel methods for analysing WGBS for organisms with non-CG methylation sites and organisms with low levels of methylation need further development. For example, methylation sites assigned by binomial distribution works well for genomes with high levels of methylation, but the accuracy of this method is lower when the number of reads is small and the level of methylation is low (Huh *et al.* 2014). This weakness may lead to underestimation of DNA methylation. Bayesian probability methods have been proposed to minimize classification errors, and possibly increase the number of reliable 5mC sites found within a genome (Huh *et al.* 2014). Lastly, a direct correlation between DNA methylation sites and their downstream effects can be monitored by RNA sequencing. Our analysis unmasked a novel metabolite and if a global metabolomics approach is used, we likely will uncover many more metabolites that are controlled by DNA methyltransferases, similar to what was discovered with histone methyl transferases.

The primary objective of this work was to develop an understanding of potential roles for DNA methylation in *F. graminearum*. We identified two DMTs and genetic analyses showed that they are involved in diverse phenotypes including fungal development, pathogenicity and the production of fungal SMs. By WGBS, we also demonstrated that DNA methylation is present in *F. graminearum* at low levels. However, this DNA methylation may be sufficient to play an important role in the regulation of

environment specific genes, leading epigenetic phenotypes. Future research is required to understand the mechanisms relating DNA methylation and the observed phenotypes.

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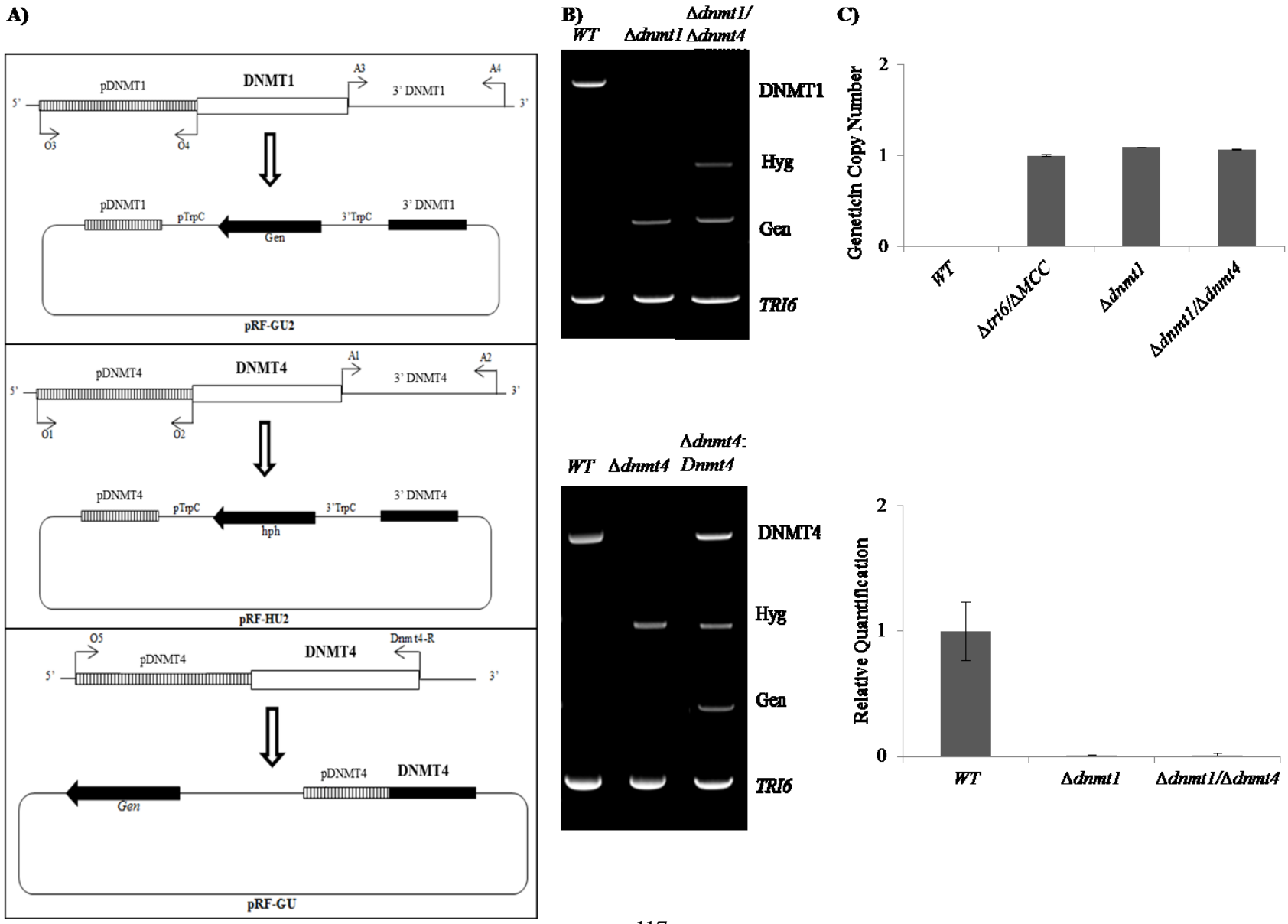
Appendix 1 Supplemental Figures

Supplemental Figure F1 (next page): Targeted Gene Deletion and Complementation of *DNMT*s in *F. graminearum*. A) Schematic depiction of gene deletion and complementation. B) PCR confirmation of gene deletion in Δ*dnmt1* and Δ*dnmt1*/Δ*dnmt4*, as well as Δ*dnmt4* and Δ*dnmt4:dnmt4*. C) Geneticin copy number by qRT-PCR compared to positive control Δ*tri6*/Δ*mcc*. D) Expression profile of *FGSG_10766* in *WT*, Δ*dnmt1* and Δ*dnmt1*/Δ*dnmt4* by qRT-PCR.


A)
pDNMT1
DNMT1
A3
A4
3' DNMT1
5'
3'
O3
O4
pDNMT1
pTrpC
3'TrpC
3' DNMT1
Gen
pRF-GU2
pDNMT4
DNMT4
A1
A2
3' DNMT4
5'
3'
O1
O2
pDNMT4
pTrpC
3'TrpC
3' DNMT4
hph
pRF-HU2
O5
pDNMT4
DNMT4
Dnmt4-R
5'
3'
pDNMT4
DNMT4
Gen
pRF-GU
B)
WT
Δdnmt1
Δdnmt1/ Δdnmt4
DNMT1
Hyg
Gen
TRI6
WT
Δdnmt4
Δdnmt4: Dnmt4
DNMT4
Hyg
Gen
TRI6
C)
Geneticin Copy Number
2
1
0
WT
Δtri6/ΔMCC
Δdnmt1
Δdnmt1/Δdnmt4
Relative Quantification
2
1
0
WT
Δdnmt1
Δdnmt1/Δdnmt4

Appendix 2 Supplemental Tables

Supplemental Table T1: Primers used in the study

Primer name	Sequence (5' to 3')
Primers used in the construction of *F. graminearum* mutants	
O1 DNMT4	gggtttaaugtggattagtgtgttgaggaac
O2 DNMT4	ggacttaauccaaggtaggtagcaacgaatg
A1 DNMT4	ggcattaautgattgaggcgcggagtatc
A2 DNMT4	ggtcttaauaggaaatgaaggagcccgtg
O3 DNMT1	gggtttaaugcagcctatcctcatgaagtga
O4 DNMT1	ggacttaaugtcctatgttaatgaatatgcacc
A3 DNMT1	ggcattaaugtcggaaacagtgtcgcgc
A4 DNMT1	ggtcttaauaccccaacatcgtttc
O5 DNMT4	ggacttaaugtgaacatggatttctgatc
DNMT4-R	gggtttaauttaggtcaattcgatc
DNMT4 F	caagacgaagcaaagctca
DNMT4 R	atgtacggatgcatgagtgt
DNMT1 F	gtggtcgatctgagccttgc
DNMT1 R	gttccgccattggatcac
HYG F	agctgcgccgatggtttctacaa
HYG R	gcgcgtctgctgctccatacaa
Gen F	tcatcaatcccagccttttc
Gen R	cagtcgatgaatccagaaaagc
TRI6 ORF F	atgatttacatggaggccg
TRI6 ORF R	acacttatgtatccgcctatagtg
Primers used in qRT-PCR analysis	
FGSG_09530 F (*β tubulin*)	gttgatctccaagatccgtg
FGSG_09530 R	catgcaaatgtcgtagaggg
DNMT1 qPCR F	ggaggtggtatcgcgatgcg
DNMT1 qPCR R	tcgatcgagcccaagaatgg
Primers used in Methylation PCR	
MSP TRI6p R	tccttccttatcttaccaaaaacacc
NMSP TRI6p R	tccttccttgtcttgccaagggcacc
MSP 1 TRI6p F	ggatggttttgtatagaagatagtttcg
NMSP 1 TRI6p F	ggatggtcttgcacagaagacagcctcg
MSP 2 TRI6p F	ggaagcgttttgttaggaa
NMSP 2 TRI6p F	ggaagcgctctgttaggaa
MSP 3 TRI6p F	gtcgtcgtattaaattgtaaatagg
NMSP 3 TRI6p F	gccgccgcatcaaacttgccaagggcacc