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Role of Silencing RNA fgsiR34 in Fusarium Graminearum's Pathogenicity to Wheat

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ROLE OF SILENCING RNA *fgsiR34* IN *FUSARIUM GRAMINEARUM*'S

PATHOGENICITY TO WHEAT

BY

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A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Sciences

Specialization in Microbiology

South Dakota State University

2016

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Sciences and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGEMENTS

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ABBREVIATIONS

15-ADON   15-acetyl deoxynivalenol

3-ADON   3-acetyl deoxynivalenol

5hmc   5-hydroxy methyl cytosine

5mc   5-methyl cytosine

ABI   Applied Biosystems

AFLP   amplified fragment length polymorphism

AFP   antifungal proteins

AGO   Argonaute

Amp   ampicillin

ATP   adenosine tri-phosphate

BLAST   Basic Local Alignment Search Tool

bp   base pair

cDNA   complementary DNA

CMC   carboxymethyl cellulose

Ct   cycle threshold

CWDEs   cell wall degrading enzymes

DCL-2   Dicer like-2

DCR	Dicer
disiRNAs	dicer-independent short interfering RNAs
DMSO	dimethyl sulfoxide
DNA	deoxyribo-nucleic acid
DNMTs	DNA methyl transferases
dNTPs	deoxyribonucleotide triphosphate
DON	deoxynivalenol
dpi	days post inoculation
dsRNAs	double stranded RNAs
E-PAP	*Escherichia coli* poly(A) polymerase
FDK	Fusarium diseased kernels
*Fg*4	*Fusarium graminearum* isolate 4
fgsiR34	*Fusarium graminearum* small interfering RNA 34
FHB	Fusarium head blight
GFP	green fluorescent protein
gpdA	glyceraldehyde 3-phosphate dehydrogenase promoter of *Aspergillus nidulans*
hph	hygromycin phosphotransferase

hpi	hours post inoculation
IPTG	isopropyl β-D-1-thiogalactopyranoside
IRT	Inverse Repeat Transgene
LB	Luria-Bertani
MAS	marker-assisted selection
MeCP2	methyl-CpG associated domain-containing protein 2
milRNAs	micro RNA-like RNAs
miRNAs	micro RNAs
MSP	methylation-specific PCR
NCBI	National Center for Biotechnology
ncRNAs	non-coding RNAs
NIL	near isogenic line
NIV	nivalenol
nt	nucleotide
PCR	polymerase chain reaction
PDA	potato dextrose agar
PEG	polyethylene glycol

ppm   parts per million

PvPGIP2   polygalactouronase-inhibiting protein

QDE-2   Quelling Deficient-2

qPCR   quantitative PCR

QTL   quantitative trait loci

RAPD   random amplified polymorphic DNA

RdRP   RNA-dependent RNA polymerase

RFLP   restriction fragment length polymorphism

RHA   RNA helicase A

RISC   RNA-induced silencing complex

RITS   RNA-induced transcriptional silencing

RM   regeneration medium

RNA   ribonucleic acid

RNAi   RNA interference

ROS   reactive oxygen species

RsAFP2   *Raphanus sativus* antifungal protein 2

RT   reverse transcriptase

RT-PCR   reverse transcriptase polymerase chain reaction

RT-qPCR   quantitative RT-PCR

SAR   systemic acquired resistance

siRNA   small interfering RNA/silencing RNA

sRNAs   small RNAs

SSR   simple sequence repeats

STC   Sorbitol+ Tris-HCl+ $CaCl_2$

tlp-1   thaumatin like protein-1

TRBP   transactivation-responsive RNA binding protein

Tri   trichothecene

trp C   tryptophan C

UDG   uracil-DNA glycosylase

US FDA   United States Food and Drug Administration

USWBSI   United States Wheat and Barley Scab Initiative

V8   Vegetable 8

YEPD   yeast extract peptone dextrose

ZEA   zearalenone

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ABSTRACT

ROLE OF SILENCING RNA *fgsiR34* IN *FUSARIUM GRAMINEARUM*'S

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Fusarium graminearum is an ascomycetous fungal pathogen that causes

Fusarium head blight (FHB) disease in wheat and other cereal grains. Mycotoxin

produced by the fungus, predominantly deoxynivalenol (DON), is considered as an

important virulence factor for the spread of disease. Our previous study of a *Dicer-like 2*

knockdown mutant has led to our hypothesis that a silencing RNA, *fgsiR34*, might play a

key role in regulating DON biosynthesis and some other virulent factors. To test this

hypothesis, we generated an *fgsiR34* over-expressing mutant (*ΔfgsiR34+*) using Inverse

Repeat Transgene method and studied the pathogenicity of the mutant in wheat. Though

no phenotypic alterations, such as spore production and growth rate on solid media, were

found in the mutant in comparison with the wildtype strain, altered expressions of *Tri*

genes and other pathogenic genes were observed. *Tri4*, *Tri5*, *Tri6*, *Tri10*, and *Tri14* were

all significantly downregulated, while the cell wall degrading enzymes (CWDEs) were

upregulated in *ΔfgsiR34+* strain. Wheat spikelets inoculated with *ΔfgsiR34+* showed a

significant downregulation of both *Tri5* and *Tri6*. The disease progression and *F.*

graminearum biomass were significantly reduced in *ΔfgsiR34+*-inoculated FHB-

susceptible NIL compared to the wildtype-inoculated ones. To understand the mechanism

of the pathogenic role by *fgsiR34*, we analyzed the methylation pattern of the seed region

of *fgsiR34* at nearly 1000 bp upstream of *Tri5* to elucidate if *fgsiR34* induces methylation

to suppress expression of *Tri* genes. After bisulfite treatment and methylation-specific PCR we found that the seed region of *fgsiR34* in both the wildtype and the *ΔfgsiR34+* strains was not methylated. In summary, our results suggested that *fgsiR34* negatively regulates *Tri* genes biosynthesis pathway, while positively regulates CWDEs. All these results imply a significant, complex role of *fgsiR34* in regulating *Tri* genes biosynthesis. It seems that methylation is not involved in repressing the expression of *Tri* genes. Interestingly, our RT-qPCR assay of non-coding transcript within the seed region revealed that the *fgsiR34* seed region was transcribed with increased transcript abundance in the *ΔfgsiR34+* mutant over the wildtype, suggesting a role of the non-coding transcript in regulating expression of *Tri* genes. More research is, therefore, needed to elucidate the mechanisms of FHB pathogenicity by *fgsiR34*.

Chapter 1 Literature Review

1.1   Introduction

Fusarium head blight (FHB), or head scab, or scab, primarily caused by *Fusarium graminearum* (sexual state *Gibberella zeae*), is a destructive disease of cereal grains such as wheat and barley. The disease development is mostly favored in moist and humid climate. The disease is so severe that a high-yielding crop can be completely destroyed within a few weeks of harvest (McMullen et al. 1997). The losses are mainly associated with reduced yields, shriveled grains, mycotoxin contamination, and reduction in seed quality (Parry et al. 1995; McMullen et al. 1997). On the one hand, the fungus causes direct loss of grain yield with the blight, and on the other hand, the indirect loss occurs through mycotoxin accumulation, predominantly deoxynivalenol (DON) produced by the fungus. The mycotoxins are a potent health hazard to both humans and livestock (Bai et al. 2001; Dexter et al. 2003). The sources of inoculum for the development of FHB are mainly crop debris (Sutton 1982), alternative hosts like grass and weeds (Gordon 1959), and Fusarium foot rot in cereal crops (Polley and Thomas 1991).

Because FHB is a widespread problem and its development in the host is a complex process, both the disease and the principal causative agent have been extensively studied. The genome of *F. graminearum* has been sequenced, annotated (Cuomo et al. 2007) and compared with other organisms (Ma et al. 2010). The genomic sequence and annotation of *F. graminearum* have been comprehensively completed and is available at Ensembl Fungi (King et al. 2015), providing excellent information for studying gene functions and performing comparative analysis with other species.

1.2   History of FHB study

FHB was first reported in England in 1884 by W.G. Smith. Kirchner reported the disease in wheat, oat, barley, rye, and maize in Germany in 1890 (MacInnes and Fogleman 1923). The disease was first reported in the United States in 1890 in Delaware by Chester and was called into attention in Indiana and Ohio the following year (Arthur 1891; Detmers 1892). The disease had been detected in 31 states, covering most of the central and eastern states by 1919 (Atanasoff 1920). Twenty-five percent of wheat fields surveyed in Manitoba had scab severities of 10% or greater, showing an increased incidence of FHB (Wong et al. 1992). The FHB epidemic in 1993 was the most devastating and greatly affected the Tristate areas of Minnesota, North Dakota, South Dakota, and the Canadian prairie province of Manitoba (McMullen et al. 1997). In 1995 significant levels of scab were reported from eastern regions of Illinois, Kansas, and Nebraska. There was a devastating effect of FHB on the soft red and soft white winter wheat, with epidemics in Iowa, Arkansas, Louisiana, Ohio, Indiana, Illinois, Wisconsin, Michigan, and New York, and Ontario in Canada (Munkvold 1996; McMullen et al. 1997). A regional epidemic that occurred in the United States in 2003 ravaged a lot of soft red winter wheat (USWBSI 2004). The effect of this epidemic prevailed in 62 counties with a huge economic loss (Cowger and Sutton 2005). In the years 2007 and 2008, FHB was less severe in the United States. However, the outbreaks were intense in parts of Nebraska and Kansas (McMullen et al. 2012). In 2009, FHB outbreak was severe in several parts of mid-south and southeastern states (Van Sanford 2009). FHB occurrence was reported to be at reduced levels in 2010, yet some parts of Ohio had severe FHB incidence (Lilleboe 2010). Despite the efforts made to mitigate FHB

incidences over the last decade, FHB infection and DON accumulation in grains have caused severe economic losses (McMullen et al. 2012).

1.3   Economic losses caused by FHB

In the United States various types of winter and spring wheat are grown every year in an area that covers about 29.1 million hectares. In 1919, scab caused an estimated loss of 2.18 million metric tons of winter and spring wheat throughout the United States (Dickson and Mains 1929). In 1982, scab caused an estimated 4% reduction in total United States wheat production (Boosalis et al. 1983). In 1991, the soft red winter wheat areas of Midwestern, Southeastern, and Mid-Atlantic States endured loss of 2.72 million metric tons with the climatic condition that favored scab development (Kephart 1991). Since 1991, many scab outbreaks have severely affected yield and quality of wheat produced (McMullen et al. 1997). The outbreaks are usually common in warmer and humid weather conditions (Schroeder and Christensen 1963; Wilcoxson et al. 1992). The scab that struck in 1993 in the tri-state areas was so serious that producers suffered an estimated $1 billion loss, one of the greatest losses in North America in a single year due to any plant disease. The epidemic affected 4 million hectares in the United States (Busch 1995; McMullen et al. 1997). There were severe yield and quality losses in grains, and mycotoxin (vomitoxin) level exceeded the U.S. Food and Drug Administration (US FDA) guideline (Moore et al. 1993). From 1998 to 2002 economic losses due to FHB reached $2.7 billion in Northern and Central USA (Nganje et al. 2002). In 2003, a regional epidemic that outbroke in southeastern states of Georgia, Maryland, North Carolina, South Carolina, and Virginia costed an estimated loss of $13.6 million (Cowger and Sutton 2005). Though the occurrence of FHB was at low levels in 2007 and 2008 in

other parts of the states, Kansas alone suffered an estimated loss of $57 million (McMullen et al. 2012). In 2009, FHB was epidemic in parts of Arkansas, Kentucky, Maryland, Missouri, North Carolina, Georgia, Illinois, Indiana, Virginia, and Tennesse, and grains conatined highly unacceptable levels of DON with poor yield (Lilliboe 2009). Kansas suffered FHB probem for four consecutive years and the FHB index (incidence x disease severity/100) ranged from 2 to 10% in the affected parts of the state. In 2010, the overall impact of FHB in Kansas only accounted to be $13 million (Lilleboe 2010). In 2011, regional impacts of FHB outbreaks was observed in some states with serious losses (Lilleboe 2011).

1.4   Causal organisms of FHB

Although *F. graminearum* is the most frequently encountered causative agent of FHB in North America (Sutton 1982; Gilbert et al. 1995), several other *Fusarium* species have also been isolated from the infected small grains worldwide (Parry et al. 1995). The predominant *Fusarium* species that causes the disease in any region depends on the climate of that region (Van Eeuwijk et al. 1995). Whilst *F. graminearum* is predominant in hotter and humid regions of the world including parts of the North America, Canada, China, Australia, and Central Europe, *F. culmorum* is more common in the cooler coastal regions of Northwest Europe (Parry et al. 1995). Several other *Fusarium* species that have been isolated from infected cereal grains include *F. poae*, *F. equiseti*, *F. avenaceum*, *F. acuminatum*, *F. crookwellense*, *F. sporotrichioides*, *F. semitectum*, and *F. tricinctum* (Stack and McMullen 1985; Wilcoxson et al. 1988; de-Galich 1997; Kosiak et al. 2003). Though several species of *Fusarium* have been reported to cause the disease, the species

that are prominent throughout the world are *F. graminearum*, *F. culmorum*, and *F. avenaceum* (Parry et al. 1995).

1.5 *Fusarium graminearum* **disease cycle**

Fusarium head blight infection is mostly favored in warm and humid conditions. Wheat heads are more susceptible to infection during anthesis up through kernel development (Sutton 1982). The asexual conidia or the sexually derived ascospores of the fungus are largely dispersed by wind or rain, gain access on the exposed anthers of the flower, and thus, initiate the infection cycle (Gilbert and Fernando 2004). *F. graminearum* survives not only in the living plant tissues, but also on the dead tissues of many cereals (Xu and Chen 1993; Shaner et al. 2003). Residues remaining on the infected crops are the principal reservoir of FHB disease. Although ascospores, macroconidia, chalmydospores, and hyphal fragments all can serve as inoculum, the major inoculum that initiate epidemics are the ascospores released from the crop debris (Xu and Chen 1993; Bai and Shaner 1994; Shaner et al. 2003). Spores released from crop residues are carried initially in air currents or splashing water and are deposited in wheat florets from where they germinate and initiate infection as shown in Figure 1.1 (Trail 2009). The fungus spreads infection in the extruded anthers and then throughout the caryopsis, floral bracts, and rachis (Bai and Shaner 1994; Bushnell et al. 2003). It may also initiate infection by penetrating directly into glumes, palea, or rachilla of the wheat floret. The glumes of the infected florets develop dark-brown, water-soaked spots and therefore become blighted. The infection then spreads to other spikelets through the vascular bundles of the rachilla and the rachis. The florets eventually either fail to produce grain, or they produce poorly filled grain (Bushnell et al. 2003). Severity of

Fusarium head blight typically depends on the abundance of inoculum, moist and warm weather conditions, and anthesis of cereal crops (Bai and Shaner 2004).
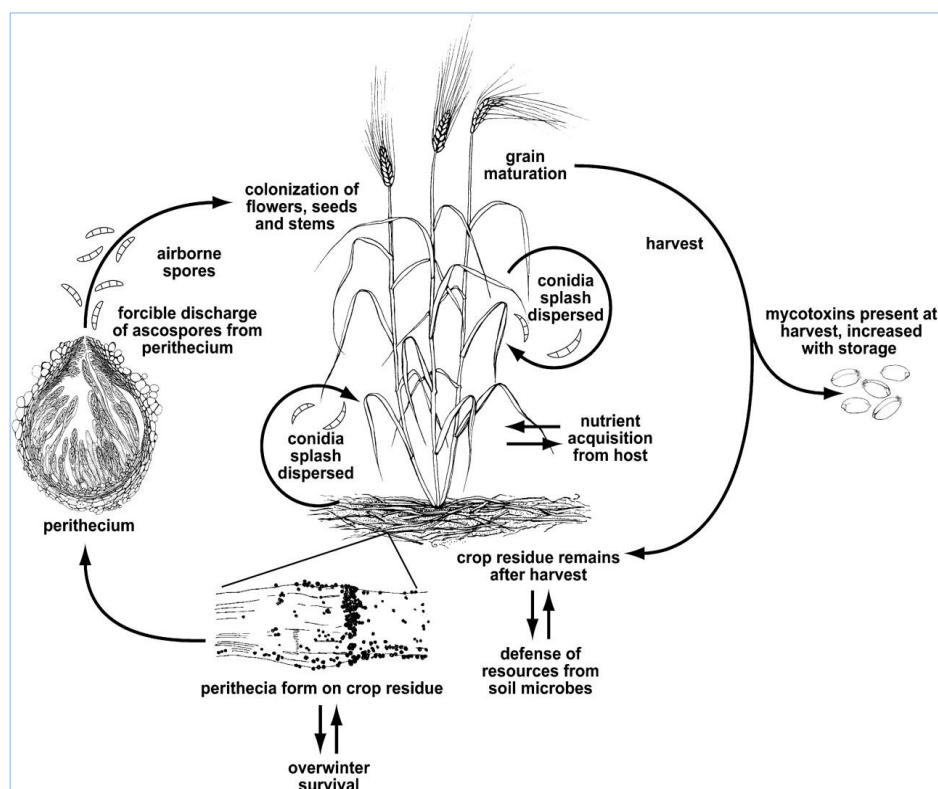
Figure 1.1 Life Cycle of *Fusarium graminearum* (from Trail, 2009).

1.6   FHB's signs and symptoms

The first visible lesions generally develop within 2 to 4 days of infection on the first florets, usually near the middle of the head (Atanasoff 1920; Andersen 1948). When temperature and moisture are favorable, lesions are water-soaked, purplish to brown colored with a bleach at the center (Tu 1930; Bennett 1931). Later they become more water-soaked and darker olive green in color and spread to the rachis (Atanasoff 1920). During prolonged infection, the fungus produces macroconidia giving pinkish tint on the surfaces of florets and glumes (Atanasoff 1920; Pugh et al. 1933). Eventually, the lesions

grow and coalesce and the entire florets become blighted (Figure 1.2). With the progress in disease, the fungus can spread up and down and horizontally in the spike (Bushnell et al. 2003; Bai and Shaner 2004). When the developing caryopsis (which matures into kernel) is infected, it produces dark brown spots that disseminate, resulting in the discoloration of the entire mature kernel. In case of severe infection, the mature kernels are completely covered with pinkish fungal mycelia producing distinct "tombstone" kernels (Bushnell et al. 2003) as shown in Figure 1.2. Generally, the effects are more severe if the infection is early (Atanasoff 1920; Andersen 1948). Even the size and the number of kernels in wheat decrease if the infection occurs during early anthesis than after late anthesis (Andersen 1948).

Diseased kernels   Healthy kernels

FHB on wheat spikelets

Figure 1.2 Symptoms of FHB on wheat spikelets and harvested kernels

1.7   *F. graminearum* **produces mycotoxins**

In addition to losses in yield of grains, the fungus produces mycotoxins in infected grains. These mycotoxins are secondary metabolites and are recognized as a health hazard for both humans and animals (Mankevičienė et al. 2007). The presence of the mycotoxins in the host depends on several factors, such as fungal strain, climatic and geographical conditions, susceptibility level of host plants, cultivation techniques, and crop protection during storage (Pancaldi et al. 2010). DON is the most commonly studied and the most important mycotoxin produced by *F. graminearum*. It has been observed to show emetic effects on humans after consumption (Perkowski et al. 1990). While low concentrations of DON in food can induce loss of appetite in animals, higher concentrations induce vomiting (Bennett and Klich 2003). The US FDA has set the maximum acceptable DON levels for human consumption in wheat grain from 0.5 to 2 ppm in the United States. Canada and some European countries follow the same limits (Dexter et al. 2003; Shaner et al. 2003). Since the mycotoxin is able to withstand high temperatures, contaminated wheat cannot be rendered safe (Hughes et al. 1999). DON is known to inhibit protein synthesis by binding to the 60S subunit of eukaryotic ribosomes, and interferes with peptidyl transferase (Ehrlich and Daigle 1987). In addition to DON, *F. graminearum* produces other mycotoxins, namely, 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), and nivalenol, as well as the phytoestrogen zearalenone (ZEA) (Yoshizawa and Morooka 1973; Greenhalgh et al. 1983; Miller et al. 1983; Pestka et al. 1985; Abbas et al. 1986).

Chemically, DON is a member of the trichothecenes family of mycotoxins (Nagy et al. 2005). Trichothecenes are a large group of terpene-derived secondary metabolites

produced by several genera of fungi, including *Fusarium, Myrothecium*, *Stachybotrys*, *Cephalosporium*, *Trichoderma*, and *Trichothecium* (Sharma and Salunkhe 1991; Bennett and Klich 2003). Besides the threat of trichothecenes to humans and animals and their phytotoxicity to cereal crops, they also act as virulence factors in head blight of maize and wheat caused by *F. granimearum* (Desjardins et al. 1996; Proctor et al. 1997; Harris et al. 1999; Bai et al. 2002; Jansen et al. 2005). Because of their widespread occurrence in cereal grains, trichothecenes are economically important mycotoxins (Cast 2003). Therefore, it is an urge to have a better understanding of the pathways and genes that regulate the biosynthesis of these mycotoxins.

1.8   DON Biosynthesis Pathway

DON is an end product of trichothecenes biosynthesis pathway (Desjardins et al. 1993). DON biosynthesis pathway and the *Tri* genes involved in its regulation have been well identified in *F. graminearum* (Brown et al. 2001; 2002; Lee et al. 2002). The *Tri* genes are positioned as more than one gene clusters in *F. graminearum* genome (Kimura et al. 1998b; Brown et al. 2001; Jurgenson et al. 2002; Kimura et al. 2003a; Meek et al. 2003; Alexander et al. 2004). A core cluster consists of twelve genes within a 25 kb *Tri5* cluster in chromosome 2 (Figure 1.3) (Kimura et al. 2003b).
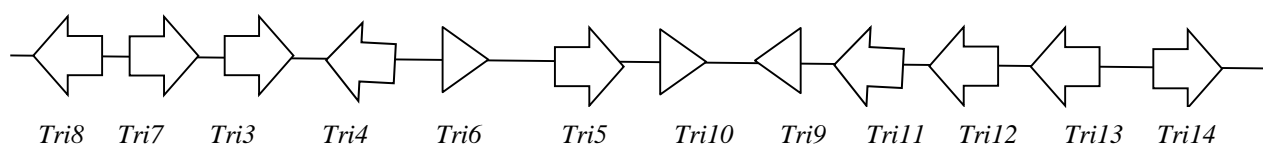
Figure 1.3 Structure of the core *Tri* cluster on chromosome 2 of *F. graminearum*

In the core cluster, *Tri5* gene encodes trichodiene synthase (sesquiterpene synthase) which catalyzes the first reaction of DON biosynthesis pathway, i.e. cyclization of farnesyl pyrophosphate to trichodiene (Hohn and Beremand 1989; Desjardins et al. 1993). There are reports that disruption of *Tri5* gene in *F. graminearum* impairs the first committed step of trichothecene biosynthesis and hence DON production (Proctor et al. 1995a; Desjardins and Hohn 1997). *Tri6* and *Tri10* are regulatory genes that control the expression of all other *Tri* genes (Proctor et al. 1995b; Tag et al. 2001; Peplow et al. 2003b). *Tri6* and *Tri10* deletion mutants showed reduced pathogenicity and toxin production in *F. graminearum* strain PH1 (Seong et al. 2009). *Tri3* and Tri7 genes encode acetyl transferase (McCormick et al. 1996; Lee et al. 2002). *Tri4*, *Tri11, and Tri13* encode P450 monooxygenase (Alexander et al. 1999; Hohn et al. 1999; McCormick et al. 2006a; Tokai et al. 2007). *Tri8* encodes esterase (McCormick and Alexander 2002). Although *Tri9* is located in the core cluster no known function of this gene in trichothecene biosynthesis has been reported (Proctor et al. 2009). *Tri12* is the transporter gene that encodes a trichothecene efflux pump. Disruption of *Tri12* gene in *F. sporotrichoides* showed both reduced growth in culture medium as well as reduced level of trichothecnene production (Alexander et al. 1999). *Tri14* encodes trichodiene oxygenase and it has been reported that the gene is required for increased virulence and DON production on wheat but not for *in vitro* DON production (Dyer et al. 2005).

Besides the *Tri5* core cluster, there are two mini *Tri* gene clusters that encode trichothecene biosynthetic enzymes. One of these mini clusters consists of a single gene *Tri101* located on chromosome 4 that encodes acyl transferase. This gene is responsible for esterification of acetate to hydroxyl function at carbon atom 3 of trichothecenes as

shown in Figure 1.4 (Kimura et al. 1998a; McCormick et al. 1999; Gale et al. 2005; Alexander et al. 2009). The second mini cluster consists of two genes, *Tri1* and *Tri16,* on chromosome 1. *Tri1* encodes a P450 monooxygenase, while *Tri16* encodes an acyl transferase (Brown et al. 2003; Meek et al. 2003; Peplow et al. 2003a). However, in *F. graminearum Tri16* is non-functional due to the presence of frameshifts and stop codons in its coding region (Brown et al. 2003; McCormick et al. 2004; McCormick et al. 2006b). The pathway for DON biosynthesis is shown in Figure 1.4.
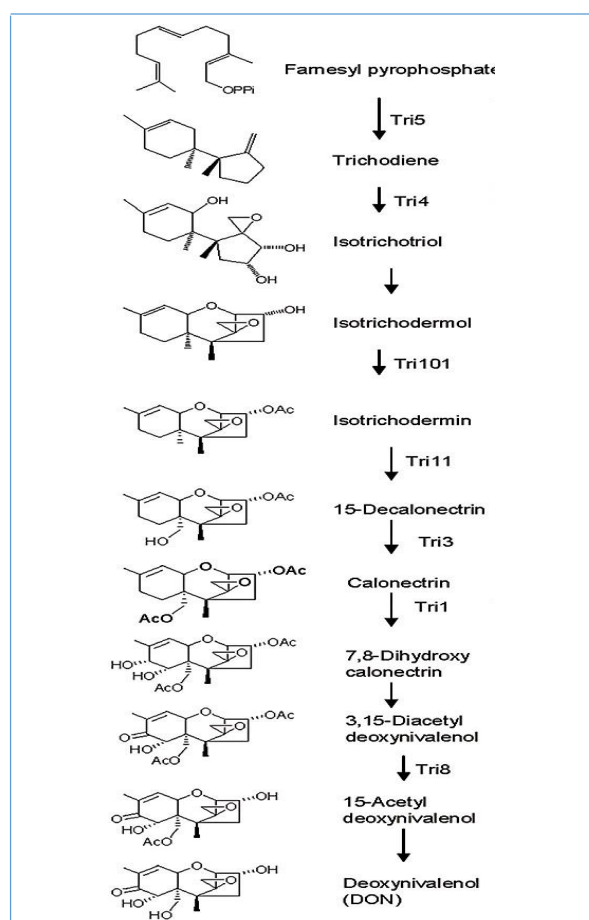
Figure 1.4 DON biosynthesis pathway showing genes encoding enzymatic steps (from Seong et al. 2009)

1.9   Cell wall degrading enzymes in *F. graminearum*

Many fungal phytopathogens are known to secrete various extracellular enzymes that can degrade the plant cell wall components and aid in host tissue infection. These enzymes are known as cell wall degrading enzymes (CWDEs). They are considered as important virulence factors and may assist in pathogenesis by degrading wax, cuticle and cell walls of host (de Vries and Visser 2001; Wanjiru et al. 2002). Secretion of these enzymes contribute to the penetration and colonization of host tissue (Jenczmionka and Schäfer 2005). Various enzymes responsible for degrading cell walls have been identified in *F. graminearum* (Phalip et al. 2005). Studies have revealed that *F. graminearum* produces cellulase, xylanase, and pectinase that aid in infection by penetrating and colonizing the wheat spike tissues (Kang and Buchenauer 2000a; Kang and Buchenauer 2000b; Wanjiru et al. 2002; Kang et al. 2005). Cytological studies conducted by Wanyoike M. Wanjiru and colleagues on wheat spikes infected by *F. graminearum* revealed degradation of host cell wall components such as cellulose, xylan, and pectin suggesting CWDEs play a role during penetration and disease establishment (Wanjiru et al. 2002). Pectinases are the first enzymes that are secreted when the fungi infect host tissues (De Lorenzo et al. 1997; Idnurm and Howlett 2001). The action of pectinases modify cell wall structure and make the components of cell wall more prone to degradation by other CWDEs (Panda et al. 2004). When gene encoding lipase was disrupted in *F. graminearum*, a reduced lipase activity in culture and decreased virulence in both maize and wheat was observed suggesting role of lipase in virulence (Voigt et al. 2005). There are also reports that suggest involvement of cutinase in the penetration of host surfaces thereby facilitating the infection (Kang and Buchenauer 2000a; Feng et al.

2005; Kang et al. 2005). *Fusarium* spp. causing FHB infection in barley contribute to β-glucanase, xylanase, and proteinase activities of grain and subsequently affect the quality of malt (Schwarz et al. 2001; Schwarz et al. 2002).

1.10   FHB resistance in wheat

Two types of FHB resistance in wheat have been generally recognized (Schroeder and Christensen 1963): Type I resistance or resistance to initial infection and Type II resistance or resistance to spread of infection within a spike. Type II resistance is common in wheat cultivar (Bushnell 2002), while Type I resistance is more common in barley (Steffenson et al. 2003). Different inoculation methods have been used in wheat to distinguish the two types of resistance. Type I resistance can be detected by spraying wheat heads with spore suspension and counting the blighted spikelets post inoculation. Type II resistance can be detected by inoculating conidia into a single floret of a spike and counting the diseased spikelets (Bai and Shaner 2004). Three other types of physiological resistance have also been proposed, including resistance to kernel infection, resistance to DON accumulation, and FHB tolerance (Miller et al. 1985; Mesterhazy 1995). The Chinese wheat cultivar Sumai 3 and its derivatives exhibit excellent type II resistance and have been widely used as source of FHB resistance in wheat breeding programs worldwide (Kolb et al. 2001; Bai and Shaner 2004). Other wheat cultivars that are often used as parents for breeding include Frontana and Encruzilhada from Brazil as they have also been reported to possess FHB resistance (Gilbert et al. 1997; Bai and Shaner 2004). Some United States breeding programs use cultivars Ernie and Freedom considering their low disease incidence and severity in the field (Rudd et al. 2001). However, FHB resistance in wheat is a complex, quantitative trait that may often be

associated with undesirable agronomic traits such as small heads, tall stature, and late maturity (Bai and Shaner 2004).

1.11   QTL for FHB resistance in wheat

A great number of quantitative trait loci (QTL) mapping studies have been conducted to identify genetic regions of wheat that confer resistance to FHB. More than a hundred QTLs for FHB resistance have been reported on all chromosomes of wheat, with the exception of 7D (Buerstmayr et al. 2009). A major QTL that confers type II resistance to FHB is located on the short arm of chromosome 3B (3BS) and has been named *Qfhb1* (also designated as *Qfhs.ndsu-3BS* or *Fhb1*). *QFhb1* possibly restricts the spread of disease within a spike and DON accumulation in harvested grains (Buerstmayr et al. 2002; Bourdoncle and Ohm 2003; Buerstmayr et al. 2003). Another QTL on chromosome 5A, *Qfhs.ifa-5A*, mainly contributes to type I resistance (Buerstmayr et al. 2003). Sumai 3 contains a QTL located on chromosome 6BS, *Fhb2*, that confers field resistance (Anderson et al. 2001; Cuthbert et al. 2007). QTL located on chromosomes 7A, 3B, 2B, and 6B from Sumai 3 significantly reduce symptoms of FHB (Zhou et al. 2002). Notably, a QTL located on chromosome 2DS in Sumai 3 has been reported to increase susceptibility to FHB. This undesirable allele in 2DS might be responsible for the lack of complete FHB resistance in many Sumai 3 derivatives (Basnet et al. 2012).

Marker-assisted selection (MAS) that uses different molecular markers to validate the genes for FHB resistance has been an important tool for breeders. The DNA segments that are genetically linked to FHB resistance genes are selected using the molecular markers (Anderson et al. 2001). Random amplified polymorphic DNA (RAPD),

restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), and amplified fragment length polymorphism (AFLP) are the commonly used methods to identify the QTL for FHB resistance (Bai and Shaner 2004). They are useful in genotyping and have varying degrees of efficiency and limitations associated in terms of cost, time and reliance on DNA sequence information.

1.12   Breeding for resistance

The most effective method of controlling FHB in wheat is the development of resistant varieties (Christensen et al. 1929; McMullen et al. 1997). Spring wheat genotypes, such as Sumai 3, Nobeoka Bozu, Shanghai 7-31B, Nyubai, Fan 1, Ning 8343, Ning 7840, Pekin 8, Frontana, Encruzilhada, are widely used as sources of FHB resistance. The conventional breeding approach to develop FHB resistant cultivar involves crossing of a resistant cultivar with a susceptible but agronomically superior cultivar (Bai et al. 1989). Nevertheless, resistance to FHB is complicated due to polygenic control of disease resistance, environmental effects on resistance phenotype, and also many possible undesired agronomic traits in the FHB resistant sources. Breeding wheat cultivars with the desired agronomic traits and a high level of FHB resistance poses a great challenge for wheat breeders (Bai and Shaner 2004). Since only a few cultivars have a high degree of resistance and they still bear some undesirable agronomic traits, crossing between moderately resistant or moderately susceptible wheat cultivars may yield transgressive segregates with better resistance and desired agronomic traits (Wang et al. 1989; Liu et al. 1991). The cultivar Sumai 3 itself was selected from a cross between two moderately susceptible cultivars, Taiwanxiaomai (a Chinese cultivar) and Funo (an Italian cultivar) (Liu and Wang 1990). A high degree of resistance to FHB with

all the desired agronomic characters including resistance to other plant diseases and insects still poses a great challenge (Bai and Shaner 2004).

1.13 Transgenic approaches for resistance

Besides the standard breeding technique, transgenic approaches have also been used to enhance FHB resistance in wheat. Since FHB resistance in the germplasm of wheat is unable to provide complete resistance from the disease and incorporating the resistance through breeding is often arduous. Methods for introducing alien resistance genes for transforming wheat have improved rapidly in recent years (Muehlbauer et al. 2003). The transgenic strategies enable the use of diverse genes that can add to FHB resistance in wheat (Bai and Shaner 2004). A few genes that are promising against fungal disease and have been used as transgenes include genes for antifungal proteins (AFP), genes regulating systemic acquired resistance (SAR), the gene for trichothecene acetyltransferase from *Fusarium* spp., and genes limiting apoptosis. Delayed FHB symptoms was reported in transgenic wheat plants that carried a rice thaumatin-like protein gene (Chen et al. 1999), maize ribosome inactivating protein b-32 (Balconi et al. 2007), bean polygalactouronase-inhibiting protein (PvPGIP2) (Ferrari et al. 2012). Similarly, increased FHB resistance was observed in transgenic wheat that expressed radish defensin (RsAFP2) (Li et al. 2011). Type II FHB resistance was conferred in FHB susceptible wheat cultivar Bobwhite after expression of the *Arabidopsis thaliana* NPR1 gene (Makandar et al. 2006) and barley class II chitinase gene (Shin et al. 2008). Furthermore, transgenic wheat overexpressing defense response genes α-1-purothionin, thaumatin-like protein 1 (tlp-1), and β-1,3-glucanase showed increased resistance to FHB (Mackintosh et al. 2007). However, there are several limitations of the transgenic

approach in terms of time, cost, and transformation efficiency which is about 0.3 to 4.3% in wheat (Cheng et al. 1997). Moreover, identification of candidate transgenes is difficult because our understanding of FHB resistance is incomplete (Muehlbauer et al. 2003).

1.14   Small RNAs and RNA interference in fungi

Small RNAs (sRNAs) are RNA species of 20-30 nucleotides (nt) in length, frequent in eukaryotes, with critical role in RNA silencing or translation repression. Many classes of small RNAs have been identified and described in eukaryotes, including fungi, based on their precursor RNA molecule and biogenesis. sRNAs are mainly categorized into two major classes- micro RNAs (miRNAs) and short interfering RNAs (siRNAs). In terms of biogenesis, both miRNAs and siRNAs are derived from cleavage of long double stranded RNA (dsRNA) precursors by Dicer to produce 21-24 nt short duplexes. miRNAs are derived from miRNA-encoding genes that generate single-stranded RNA precursor transcripts containing fold-back or hairpin structures. miRNAs target mRNAs that do not have perfect match leading to target cleavage or translation repression (Ambros et al. 2003; Bartel 2004; Chen 2009).

In contrast, siRNAs are derived from perfectly complementary long double stranded RNA (dsRNA) molecules (Bartel 2009; Katiyar-Agarwal and Jin 2010). There are various ways by which the trigger molecule, dsRNA, can be derived including simultaneous sense and antisense transcription of specific genomic loci, repetitive sequences that have foldback-structured transcripts, and intermediates from viral replication (Saito et al. 2010). RNA interference comes into play when an RNase III enzyme (Dicer) cleaves dsRNA molecules into short 21-25 nt duplexes (Bernstein et al. 2001). These short siRNA duplexes, later unwind. The sense strand referred to as the

passenger strand is degraded, while the anti-sense strand which is complementary to the target called the guide strand is incorporated into RNA-induced silencing complex (RISC). RISC consists of a protein called Argonaute 2 (AGO 2) that targets the homologous RNA and cleaves it at specific site resulting in the mRNA degradation and suppression of the target gene expression (Elbashir et al. 2001; Hammond et al. 2001). Besides, additional factors that interact in the RNAi pathway include RNA helicase A (RHA) that is associated with RISC, transactivation-responsive RNA binding protein (TRBP) and Dicer (Robb and Rana 2007).

Although myriad of studies on sRNAs have been made in higher eukaryotes such as plants and animals, the studies in fungi have diversified sRNA pathways in eukaryotes. After the discovery of quelling, a transgene-induced silencing phenomenon, in the filamentous fungus, *Neurospora crassa*, (Napoli et al. 1990; Romano and Macino 1992) many fungal species have been investigated for gene silencing. Quelling detects and targets transgenes and mediates suppression of gene expression and transposon expansion (Chicas et al. 2005; Nolan et al. 2005). A set of RNA silencing proteins QDE-1 (Quelling deficient-1 as RdRP) (Cogoni and Macino 1999a), QDE-2 (AGO) (Cogoni and Macino 1999b), and DCL-2 (Dicer-like 2) (Catalanotto et al. 2004) mediate this process. Metzenberg and colleagues discovered another gene silencing phenomenon in *N. crassa*, similar to quelling, known as meiotic silencing by unpaired DNA (MSUD) (Aramayo and Metzenberg 1996; Shiu and Metzenberg 2002; Shin et al. 2008). MSUD appears to function only during meiosis and dsRNA synthesis is required for this mechanism (Shiu and Metzenberg 2002). Necrotrophic fungus, *Botrytis cineria*, employs its siRNAs as virulence factor to mediate silencing of plant defense genes (Weiberg et al. 2013;

Weiberg et al. 2014). RNA silencing has been used as a functional genomics tool in several other fungi including *Saccharomyces pombe* (Drinnenberg et al. 2009), *Aspergillus oryzae* (Yamada et al. 2007), *A. nidulans (Hammond and Keller 2005)*, *A. parasiticus*, *A. flavus*, and *F. graminearum* (McDonald et al. 2005), *Magnaporthe oryzae* (Kadotani et al. 2003), and a few filamentous fungi. A better understanding of RNA silencing mechanism and sRNA pathways in plants and fungi could help explore plant-fungal interaction as well as lead to the development of new targets for fungal control.

1.15   DNA methylation in fungi

Studies have shown the role of non-coding RNAs (ncRNAs) in regulating epigenetic phenomena (Bernstein and Allis 2005). The ncRNAs are RNA molecules transcribed from the genome segments that do not encode proteins. Many of them regulate gene expression at transcriptional or post-transcriptional levels. The two major groups of ncRNAs that appear to be involved in epigenetic regulation are short ncRNAs (less than 30 nt) and long ncRNAs (greater than 200 nt). The short ncRNAs include micro RNAs (miRNAs), short interfering RNAs (siRNAs), and piwi interacting RNAs (Iyengar et al. 2015). Both short and long ncRNAs appear to play important role in heterochromatin formation, histone modification, DNA methylation and gene silencing. siRNAs have also been shown to induce heterochromatin formation via an RNA-induced transcriptional silencing (RITS) complex which when bound to siRNA stimulates H3K9 methylation and chromatin condensation (Carthew and Sontheimer 2009).

DNA methylation plays an important role on gene function by maintaining a repressive chromatin structure, employing methyl binding proteins, and interfering with transcription binding factor (Curradi et al. 2002; Havliš and Trbušek 2002). Therefore,

detecting the methylation status of the cytosine residues in the genome has become crucial to understand variety of cellular processes essential for normal development.

The methylation status of promoter regions of individual genes can be detected by methylation-specific PCR (MSP) (Herman et al. 1996). This method is based on the bisulfite modification of genomic DNA, after which, the unmethylated cytosines are converted to uracils and to thymines after PCR, while the methylated cytosines do not undergo modification. Furthermore, two sets of primer pairs are designed to the gene of interest that anneal to sequences containing CpG dinucleotides. One primer pair specific for the sequence recognizes methylated CpGs and is designated as M primer, whereas the other primer pair specific for the sequence recognizes unmethylated CpGs and is designated as U primer. Generally, the primer pairs specific for methylated and unmethylated sequences are designed for the same gene, and the PCR reactions with the two primer sets are performed on separate tubes to amplify the target the gene. An overview of MSP is shown in Figure 1.5.

Methylated DNA   **Unmethylated DNA**

ATCCGCGGTTCCGAATCG   ATCCGCGGTTCCGAATCG

↓   **Bisulfite treatment**   ↓

ATUCGCGGTTUCGAATCG   ATUUGUGGTTUUGAATUG

↓   **PCR**   ↓

ATTCGCGGTTTCGAATCG   ATTTGTGGTTTTGAATTG
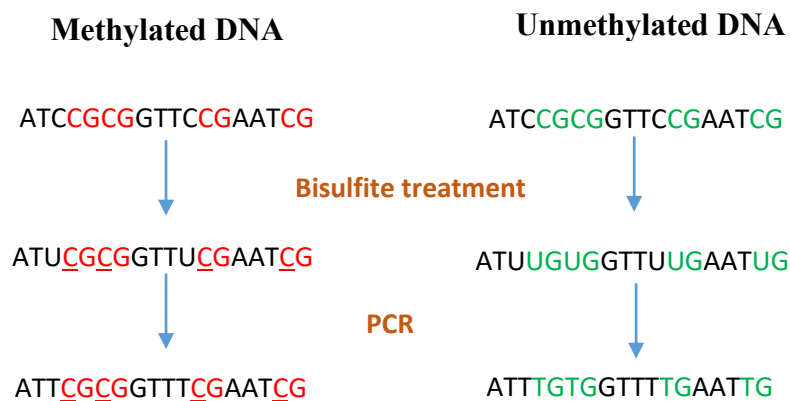
Figure 1.5 Overview of bisulfite conversion and MSP

The amplified products are then resolved on an agarose gel side by side for comparison. If the band specific for M primer set is seen, the gene of interest is considered methylated. On the other hand, if the band specific for U primer set is observed, the sequence of interest is considered unmethylated (Ohashi 2002; Huang et al. 2013). The schematic representation of the biochemical reaction pathways for conversion of unmethylated cytosine to uracil is shown in Figure 1.6.
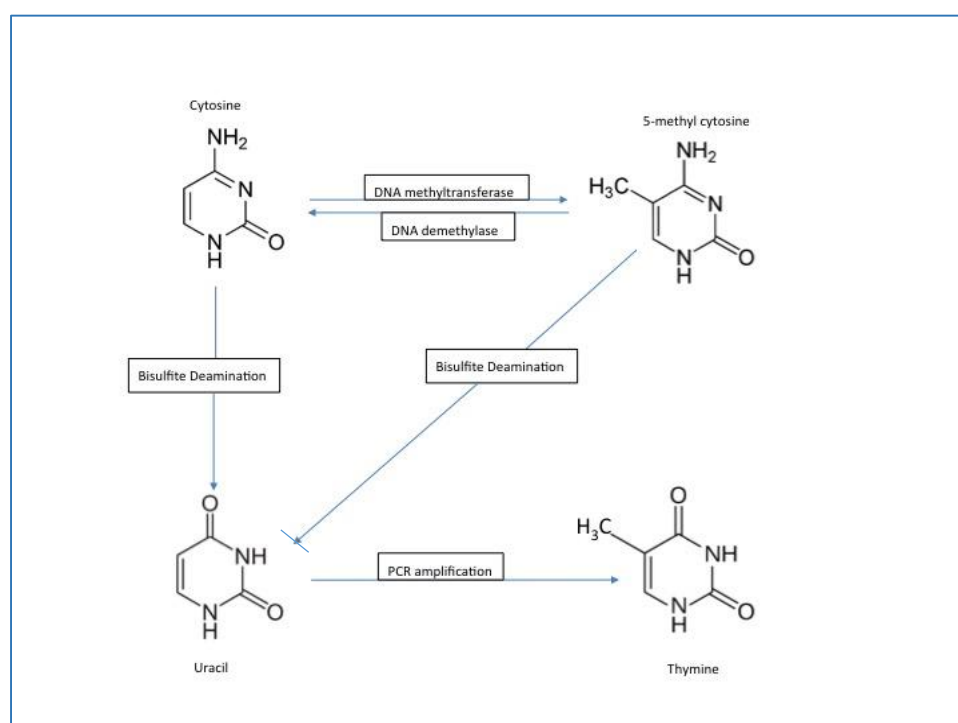
Figure 1.6 Schematic approach of conversion of cytosine to thymine by bisulfite treatment. Cytosine is converted into uracil by sodium bisulfite through a series of sulfonation, hydrolytic deamination, and desulfonation reactions, while 5-methyl cytosine is protected from the bisulfite reaction due to the presence of methyl group that hinders the sulfonation by bisulfite.

In some fungi, DNA methylation has been associated with different growth stages. Dormancy and lower transcriptional activity are associated with higher levels of methylation, while higher transcriptional activity and active growth stages have been

observed with lower levels of methylation (Russel et al. 1985; Jupe et al. 1986; Russell et al. 1987).

1.16   Strategies to manage FHB

FHB results from interaction between the host and the pathogen in a suitable environment, and disrupting this interaction should help prevent the disease. Understanding this interaction should help mitigate the effects of FHB on cereal grains. Managing FHB requires several disease management strategies. There are integrated factors that contribute to the development of FHB in crops which include moisture level and climate, high proportions of minimum tillage, cultivated acres planted to susceptible host crops, and short crop-rotation intervals between susceptible crops. Various practices have been proposed to eliminate the sources of primary inoculum (Bai and Shaner 1994). Some of these are the adoption of minimum tillage for soil conservation, seed treatment and foliar application of fungicide at anthesis (Mesterházy et al. 2003), and application of certain fungicides to cereals late in the season (Jones 2000; Shaner and Buechley 2003; Steffenson et al. 2003).

The risk of severe FHB can be reduced by crop-rotations. It has been observed that the possibility of FHB outbreak is higher if the preceding crop is susceptible to FHB (McMullen et al. 1997). This is notably true in case of corn since it produces high amount of crop residue (Leplat et al. 2013). Application of fungicides may be useful when climate is warm and humid, which favors FHB development. Best results for FHB control by fungicides have been achieved when they are sprayed at full flowering stage directly from two sided of the head (Da Luz et al. 2003; Yoshida et al. 2012). Though the use of fungicide minimizes direct yield loss, the level of mycotoxin in cereals is often not

acceptable for human consumption (Martin and Johnston 1982; Steffenson et al. 2003). Therefore, solely relying on fungicides for the disease control is risky.

Biological control measures have also been practiced to protect wheat heads against FHB infection. Most commonly studied bacterial strains belong to the genera of *Bacillus*, *Lysobacter*, and *Pseudomonas* (Schisler et al. 2002; Da Luz et al. 2003; Khan et al. 2004; Jochum et al. 2006). Yeast strains of *Cryptococcus* spp. have been promising biological control for managing FHB severity in wheat (Khan et al. 2004).

Despite the efforts to reduce direct yield loss due to FHB severity in cereal grains, potent mycotoxins harbor a portion of grains that make the grains unfit for consumption. Therefore, measures to restrict mycotoxin production by pathogen are of great need. One measure is using endophytic microorganisms (endophytic fungi and bacteria) as they can penetrate plant tissues and confer ecological benefits (Redman et al. 2002; Zinniel et al. 2002; Schardl et al. 2004). It has been observed that a corn endophyte, *Acremonium zeae*, reduced kernel rot and mycotoxin production by *Aspergillus flavus* and *Fusarium verticilloides* (Donald et al. 2005). Employing enzymes that inhibit and detoxify DON is another strategic measure. It has been reported that a UDP-glucosyltransferase enzyme from *Arabidopsis thaliana* has ability to detoxify DON produced by *F. graminearum* (Poppenberger et al. 2003). For optimum management of FHB and DON contamination in cereal grains, exploiting integrated measures works effectively than single strategy (McMullen et al. 2008; Blandino et al. 2012).

Chapter 2 Role of silencing RNA *fgsiR34* in *Fusarium graminearum*'s pathogenicity to wheat

2.1   Introduction

Fusarium graminearum Schwabe (teleomorph *Gibberella zeae* [Schweinitz] Petch) is one of the most devastating pathogenic fungi of cereal grains. It is the causative agent of Fusarium Head Blight (FHB) of wheat and barley and is also known to cause stalk and ear rot of maize (Bai and Shaner 1994; McMullen et al. 1997; Goswami and Kistler 2004). FHB is mostly favored in warm and humid weather conditions during anthesis up through early stages of kernel development (Parry et al. 1995; McMullen et al. 1997; Gilbert and Tekauz 2000). *F. graminearum* is a saprophyte and survives not only in the living tissues but also on the dead tissues and crop residues producing mycelia on the soil surface which act as the major reservoir of the disease (Xu and Chen 1993; Shaner et al. 2003).

Moist weather favors maturation of perithecia from which ascospores are forcibly released concomitantly with the flowering of crops (Trail et al. 2002; Markell and Francl 2003) and serve as the source of inoculum for the spread of disease to crops via air, rain, or wind (Sutton 1982; Xu and Chen 1993; Bai and Shaner 1994; Parry et al. 1995; Shaner et al. 2003). Primarily, the fungus deposited on the wheat floret spreads from one spikelet to the other through the vascular bundles in the rachis and rachilla (Ribichich et al. 2000). Under temperate and humid conditions, the fungus may also directly invade glumes, palea, or lemma (Bushnell et al. 2003). Infected wheat heads appear blighted and kernels turn out shriveled, discolored and often poorly filled (Bushnell et al. 2003; McMullen et

al. 2012). FHB infection largely reduces grain yield and quality severely affecting the market price.

F. graminearum, most importantly, contaminate the infected grains with trichothecene mycotoxins, predominantly deoxynivalenol (DON), that render the harvested grains unsuitable for food or feed (McMullen et al. 1997; Bushnell et al. 2003; Desjardins 2006). Trichothecenes are sesquiterpene epoxides that have a common tricyclic nucleus (hence the name trichothecene) and an epoxide group at C-12 and C-13 position, which is responsible for their toxicity (Gledhill et al. 1991). They generate free radicals that subsequently produce harmful levels of oxidative stress (Suneja et al. 1989; Riley and Norred 1996). Trichothecenes have been broadly classified into four types (A, B, C, and D) based on their variation in chemical structure. The two major types- Type A and Type B are predominantly produced by *Fusarium* spp. and are widely distributed in cereals as toxic metabolites (Krska et al. 2001). The two types differ at their C-8 position. Type A has either hydroxyl, or ester, or no oxygen substitution at C-8 group, while Type B has carbonyl function at C-8 position (McCormick et al. 2011). *F. graminearum* and *F. culmorum* are the most important Type B trichothecene producers (Birzele et al. 2000; Homdork and Beck 2000). Nivalenol, fusarenon-X, DON, and DON's acetylated derivatives- 3-acetyldeoxynivalenol and 15-acteyldeoxynivalenol, collectively belong to the Type B trichothecene group (Ueno and Hsieh 1985). There are two chemotypes of Type B trichothecene producers- Type I produce DON and/or its acetylated derivatives, while Type II produce NIV (Sydenham et al. 1991; Perkowski et al. 1997; Lee et al. 2001; Chandler et al. 2003).

DON is the most important and predominant mycotoxin associated with FHB infection (Gale et al. 2003). It is a potent inhibitor of protein synthesis and acts by binding to 60S ribosomal subunit of eukaryotic cells (Desjardins et al. 1993; Parry et al. 1995; McMullen et al. 1997). DON has emetic effects when ingested and therefore is also known as vomitoxin. It is associated with feed refusal and reduced growth in animals, and immune-suppression and teratogenic effects in humans (Snijders 1990; Rocha et al. 2005; Desjardins 2006).

DON biosynthesis pathway has been well characterized in *F. graminearum* (Desjardins 2006; Proctor et al. 2009). DON biosynthesis is regulated by *Tri* gene clusters located on three different chromosomes in *F. graminearum* (Cuomo et al. 2007; Lee et al. 2008). The core cluster consists of twelve genes on chromosome 2 that are responsible for the synthesis of core trichothecene molecule (Lee et al. 2008). *Tri5* encodes an enzyme trichodiene synthase that catalyzes the very first step of trichothecene biosynthesis (Proctor et al. 1995a; Bai et al. 2002). Studies have shown that mutant strains of *Tri5* exhibited reduced virulence on some cultivars of wheat (Proctor et al. 1995a). In another study of a DON non-producing strain of *F. graminearum* (generated by disrupting Tri5 gene), the fungal growth, when inoculated in wheat spikelet was limited only to the inoculated spikelet suggesting that DON is not required for initial infection but plays an important role for the spread of disease (Bai et al. 2002). More specifically, DON is required for the passage of fungi from infected florets to rachis and to wheat head for further colonization (Jansen et al. 2005). *Tri6* and *Tri10* are transcriptional regulators that control trichothecene biosynthesis (Proctor et al. 1995b; Tag et al. 2001; Peplow et al. 2003b). Mutants of *Tri6* and *Tri10* showed greatly reduced

pathogenicity and toxin production as well as altered *Tri* gene transcript levels for all the known *Tri* genes revealing global gene regulation by these genes in *F. graminearum* (Seong et al. 2009). In addition to this, there are two separate smaller loci that encode trichothecene biosynthetic enzymes- *Tri1* and *Tri16* on chromosome 1 (Brown et al. 2003; Meek et al. 2003) and *Tri101* on chromosome 4 (Kimura et al. 1998b).

Moreover, DON has been shown to elicit the production of hydrogen peroxide in wheat during infection that may lead to programmed cell death of the host and consequently necrotrophic growth of the fungus (Desmond et al. 2008). The necrotrophic stage of FHB pathogenesis favors vigorous colonization of the host tissue by the pathogen (Inch and Gilbert 2003). Wheat responds to necrotrophic fungi by inducing reactive oxygen species (ROS) such as superoxide dismutase that can neutralize free radicals (Lamb and Dixon 1997). The level of ROS was found to be comparatively higher in FHB resistant wheat cultivars compared to the FHB susceptible ones (Wang et al. 1992; Lifeng et al. 1997). Furthermore, DON is also known to inhibit thickening of cell wall and callose deposition which are the defense responses of host against the progression of the disease (Walter et al. 2010).

The discovery of sequence-specific gene silencing as a response to double-stranded RNAs (dsRNAs), by Fire and Mello in 1998, has made a remarkable impact for studying gene functions by inhibiting the expression of targeted genes. This phenomenon is known as RNA interference. Diverse small RNAs and RNAi pathways have been investigated across eukaryotic organisms (Nakayashiki 2005; Schumann et al. 2010). Small RNAs (sRNAs) are non-coding RNA molecules frequent in eukaryotes. They are 20-30 nucleotides (nt) in length and function in gene regulation via RNA interference

(RNAi) related pathways. RNAi is a conserved eukaryotic mechanism in which double-stranded RNA (dsRNA) mediates sequence-specific post-transcriptional gene silencing (Hannon 2002; Ambros 2004; Bühler and Moazed 2007; Ghildiyal and Zamore 2009). There are two main classes of sRNAs- short interfering RNAs (siRNAs) and micro RNAs (miRNAs) (Carthew and Sontheimer 2009; Ghildiyal and Zamore 2009). Both are generated from dsRNAs that are processed by ribonuclease III enzyme Dicers. However, siRNAs and miRNAs differ in their biogenesis and mode of action. siRNAs generate from exogenous dsRNAs, or endogenous transcripts from repetitive sequences, or from transcripts with long hairpins (Hannon 2002; Carthew and Sontheimer 2009; Ghildiyal and Zamore 2009). siRNAs target mRNAs that are fully complementary and mediate transcriptional silencing via Argonaute (AGO) proteins. Their major function is genomic defense. In contrast, miRNAs derive from long single stranded RNAs that fold and form imperfect hairpin dsRNAs. miRNAs do not need to match fully to target mRNAs to cause degradation and translation repression (Ambros et al. 2003; Bartel 2004).

Mechanism of RNAi has been studied in several species of fungi, including *Neurospora crassa, Magnaporthe oryzae, Cryptococcus neoformans, Aspergillus fumigatus, A. nidulans, Histoplasma capsulatum*, and *Venturia inaequalis* (Cogoni and Macino 1999a; Liu et al. 2002; Kadotani et al. 2003; Catalanotto et al. 2004; Fitzgerald et al. 2004; Mouyna et al. 2004; Rappleye et al. 2004; Hammond and Keller 2005). The filamentous fungus, *N. crassa*, has been extensively used as the model organism for the study of RNA silencing pathways since the discovery of quelling in 1992 (Romano and Macino 1992). Quelling is the post-transcriptional gene silencing mechanism in fungi triggered by introduction of transgenes and is related to RNAi (Fulci and Macino 2007).

Several fungal species that have been studied contain one or more genes for each of the three components of RNAi machinery which include Dicer-like (DCL) proteins, AGO, and RNA-dependent RNA polymerase (RdRP) (Nakayashiki 2005). RNAi functions when aberrant RNAs that are generated from repetitive transgenes are recognized by RdRps which convert them to dsRNAs. These dsRNAs are processed into 22-24 nt siRNAs by DCL proteins which are subsequently loaded onto AGO proteins that confer silencing of the target transcript (Nakayashiki et al. 2006). Though RNAi and its components appear to be conserved in most of the fungal species, the pathway seems to be lost in the budding yeast, *Saccharomyces cerevisiae* which lacks all the components of the RNAi machinery (Nakayashiki et al. 2006; Laurie et al. 2008). Despite this, the lost RNAi in *S. cerevisiae* has been reconstituted by introducing AGO1 and DCR1 proteins from its close lineage *S. castellii* (Drinnenberg et al. 2009).

This study reports our efforts to understand the role of siRNA, *fgsiR34*, in FHB pathogenesis of wheat. *fgsiR34* was identified as the only significantly downregulated siRNA in our previous study in *F. graminearum* isolate 4, *Fg*4 (a local isolate used in our lab for FHB study) when *DCL-2* gene was knocked down. In the *dcl-2* mutant, *Δdcl2-*, we also observed a significant knock down of the key *Tri* genes (*Tri4, Tri5, Tri6, Tri10*, and *Tri14*). In association with the reduced expression of the *Tri* genes, we observed a significantly reduced DON production in the *Δdcl2-* mutant inoculated grains. These results led us to hypothesize that silencing RNA *fgsiR34* might play an important role in DON biosynthesis pathway and so in FHB pathogenicity of *F. graminearum* (Galla 2014).

2.2   Materials/ Methods

2.2.1   Fungal growth, RNA extraction and cDNA synthesis

The wildtype *Fg*4 was grown on potato dextrose agar (PDA) medium using the hyphal tip inoculation method on the surface of the agar plate, and incubated at 28°C for a week in the dark. Total RNA was isolated from the fungus using TRIzol® Reagent (Life Technologies) followed by DNase I treatment (Thermo Scientific). Integrity of the isolated RNA was determined by resolving in 1% agarose to ensure that it was not degraded. The concentration of the RNA sample was determined using Nano Drop ND-1000 Spectrophotometer. First-strand cDNA synthesis was done from the total RNA (1 µg/reaction) in a 20 µL reaction using Superscript III enzyme (Promega) and a 3' adaptor. Reverse transcription was carried out at 25°C for 5 min, 42°C for 60 min and 70°C for 15 min. A two-fold dilution of the synthesized cDNA was made.

2.2.2   PCR amplification, cloning and sequencing

PCR amplification was done in a 20 µL reaction using 1 µL of the diluted cDNA, 2X GoTaq Green Master Mix, and primers spanning the sequence of *fgsiR34*. The PCR program was set at an initial denaturation temperature of 95°C for 2 min, 30 cycles of 95°C for 45 s, 55°C for 45 s, 72°C for 60 s, and a final extension of 72°C for 5 min, and then an infinite hold at 4°C. The PCR product obtained was resolved in 1% agarose and purified using the QIAquick PCR Purification kit (Qiagen).

The purified PCR product was ligated into the pGEM-T vector (Promega) using 2X Rapid Ligation Buffer and T4 DNA ligase, transformed into competent *E. coli* cells (JM 109), plated into LB/Amp/IPTG/X-Gal plates, and incubated overnight at 37°C. The

transformants were analyzed by colony PCR to confirm the presence of inserts. A single white colony was picked and grown in 2 mL of LB broth containing 100 µg/mL of ampicillin overnight at 37°C with shaking at 220 rpm. Plasmid DNA was isolated and purified using the PureLink™ HQ Mini Plasmid Purification Kit and sent for sequencing to GenScript. The sequence of the cloned fragment was validated and cloning of 204 bp fragment containing sequence of *fgsiR34* into the vector was successfully done.

2.2.3 Vector construction for over-expressing *fgsiR34*

For the vector construction, inverse repeat transgene (IRT) method (McDonald et al. 2005) was used with slight modification. Around 204 bp of the sense and the anti-sense strands containing *fgsiR34* sequence were ligated between the *gfp* spacer of the plasmid, *p*TMH44.2. This plasmid is a modified derivative of *p*AN52-3 by addition of 280-bp *gfp* spacer between gpdA promoter and trpC terminator.
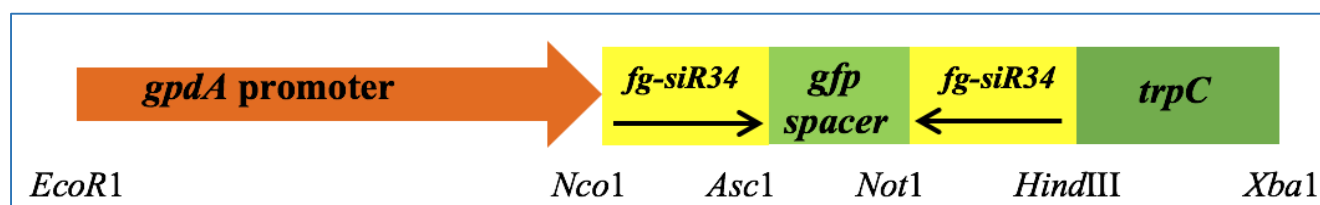
Figure 2.1 Illustration of construct for over-expressing silencing RNA *fgsiR34*.

The sense strand was generated by amplifying with a forward primer having *Nco*1 site and a reverse primer having *Asc*1 site. The amplified product was digested with the enzymes *Nco*1 and *Asc*1 and ligated in sense orientation to *Nco*1/*Asc*1 site of *p*TMH44.2. Similarly, the anti-sense strand was generated by amplifying with a forward primer having *Not1* site and a reverse primer having *Hind*III site, digested with *Not*1 and *Hind*III enzymes, and ligated in anti-sense orientation to *Not*1/*Hind*III site of the same plasmid

(Figure 2.1). The size of the plasmid after ligation of sense and anti-sense strands was around 6.35 kb. The construct *p*TMH44.2-IRT was then cut at *Xba*I.

Another plasmid, *p*UCATPH, with hygromycin resistance cassette, was used for the selection of transformants in hygromycin containing medium (Figure 2.2). The
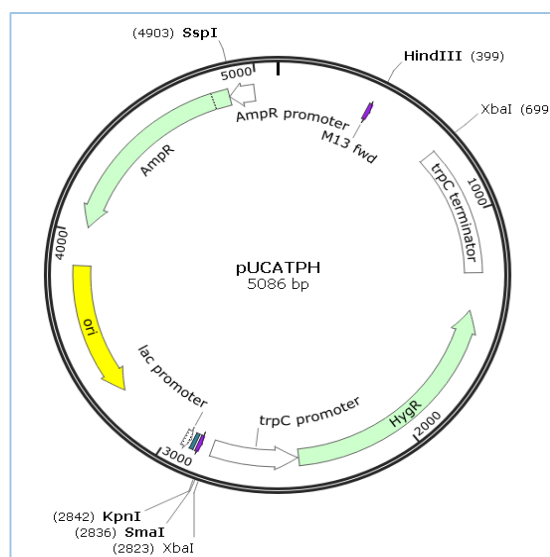
Figure 2.2 Map of vector with hygromycin resistance gene for selecting transformants

plasmid was also cut with *Xba*I and an around 2100 bp fragment containing hygromycin resistance cassette was released. This fragment was ligated to the *Xba*I site of *p*TMH44.2-IRT construct. The resulting construct, *p*TMH44.2-IRT-pUhyg, which was around 8.45 kb, was then transformed into fungal protoplasts to generate *fgsiR34* over-expression mutant (*ΔfgsiR34+*).

Construct for empty vector was also generated to study the effect of vector on gene silencing. An around 2100 bp fragment of hygromycin resistance gene along with trpC promoter and trpC terminator was released from *p*UCATPH after digestion with *Xba*I and ligated to *Xba*I site of *p*TMH44.2 generating approximately 8 kb plasmid. The

plasmid (*p*TMH44.2-pUhyg) was then transformed to *Fg*4 protoplasts to generate empty vector transformant control.

2.2.4 Fungal protoplasts generation

The protoplasts for the transformation of *Fg*4 were prepared according to Hallen-Adams et al (Hallen-Adams et al. 2011). For this purpose, discs of *Fg*4 mycelia grown on PDA medium were added onto carboxymethyl cellulose (CMC) medium for sporulation. After incubation for 6 d at 25°C and 250 rpm, the spores were transferred to YEPD (yeast extract+ peptone+ dextrose) medium for 10-14 h at 25°C and 175 rpm for germination. The germinated spores were treated with protoplasting buffer containing 1.2M KCl and the cell wall digesting enzymes, driselase (from Basidiomycetes), chitinase (from *Streptomyces griseus*), and lysing enzyme (from *Trichoderma harzianum*) for 1.5 h at 30°C and 80 rpm. This treatment temporarily removes the fungal cell wall and generates protoplasts. After subsequent centrifugation steps the protoplasts were stored in STC (sorbitol+ Tris-HCl+ CaCl2) buffer and preserved in 7% (v/v) DMSO at -80°C in 50 µL aliquots.

2.2.5 Polyethylene glycol-mediated fungal transformation

The fungal protoplasts were transformed by the polyethylene glycol (PEG) method as described by Hallen-Adams et al (2011). For this purpose, a mixture of 100 µL protoplast suspension, 100 µL STC buffer, 50 µL 30% PEG, and 10 µL 40 µg vector (pTMH-IRT-pUhyg) was made in a 15 mL Falcon tube. To this mixture, 2 mL 30% PEG was added and further incubated for 20 min at room temperature. Ice cold 4 mL STC buffer was added to this mixture and the whole reaction was poured into cooled 150 mL

regeneration medium (RM- made by mixing 135.5 g sucrose in 500 mL water, 0.5 g yeast extract, 0.5 g N-Z amine, and 3.72 g agar and autoclaving the mixture). The reaction was mixed, poured onto plates, and left at room temperature for 16 h to allow the protoplasts to regenerate. The medium was then overlaid with RM containing 150 µg/mL of hygromycin B and incubated in the dark at 28ºC for 7 d for selection of transformants. Emerging transformants were sub-cultured on V8 medium (made by mixing 163 mL V8 juice, 1 g $CaCO_3$, and 15 g agar in 1 L water and autoclaving the mixture) containing 150 µg/mL of hygromycin B for two cycles to screen the putative ones. The putative transformants were sub-cultured on PDA medium amended with 150 µg/mL of hygromycin B to establish the population.

2.2.6 DNA extraction and PCR amplification of GFP spacer and hygromycin phosphotransferase gene

Genomic DNA was extracted from one-week old *F. graminearum* strains of all three types- wildtype, *ΔfgsiR34+*, and empty vector transformant grown on PDA plates. For DNA extraction, Qiagen's DNeasy Plant Mini Kit was used following manufacturer's instructions. The quality of the extracted DNA was tested by resolving it in 1% agarose gel to assure its integrity and quantified using Nano-Drop ND-1000 Spectrophotometer.

PCR amplification was done from DNA extracted from all three strains using two different primer sets: *gfp* spacer forward and reverse primer set and hygromycin phosphotransferase (*hph*) gene forward and reverse primer set. PCR reaction was prepared by mixing 10 µL 2X GoTaq® Green Master Mix, 1 µL each of 10 µM forward and reverse primers, 1 µL (10 ng) genomic DNA and water to 20 µL. The PCR program was set at an initial denaturation temperature of 95ºC for 2 min, thirty-five cycles at 95ºC

for 45 s, 55°C for 45 s, and 72°C for 60 s, a final extension of 72°C for 5 min, and an infinite hold at 4°C. PCR products were run by resolving in 1% agarose gel and the gel was observed in ABS Imaging System.

2.2.7   Fungal inoculum preparation

All the three fungal strains, the wildtype, *ΔfgsiR34+*, and the empty vector transformant, were grown on PDA medium by hyphal tip method. The plates were covered with aluminum foil to maintain dark condition and incubated at 28°C for 7 d. For spore preparation, a pea sized mycelium was inoculated onto CMC medium and incubated in a rotary shaker at 25°C and 250 rpm for 6 days. Prior to inoculation in wheat spikelets, the spores were counted using hemocytometer and diluted to 80,000-100,000 spores/mL.

2.2.8   Spore production assay

The three strains of *F. graminearum* were analyzed for the number of macroconidia production in culture. To perform this assay, a pea sized mycelium was inoculated onto CMC medium, for 6 d at 25°C. The culture was filtered through sterile Miracloth into a conical flask and the supernatant was collected. After subsequent centrifugation steps of the supernatant, spores were pelleted and counted using hemocytometer. Four biological replicates were cultured for each strain. The number of macroconidia produced by each biological replicate was counted and averaged. The average number of macroconidia produced by all three strains was compared using t-test at $p \leq 0.05$ significance level.

2.2.9   Radial growth assay

The wildtype and *ΔfgsiR34+* strains were compared for their growth rate on solid media. For this four PDA plates were divided into two halves to maintain same condition of growth for fungal strains. Spores were counted in hemocytometer prior to inoculation and adjusted to 100,000 spores/mL. Ten µL spores of each type of fungal strain was inoculated on each half of the plate. All the plates were incubated in the dark at 28°C for 5 d. The mean diameter of the four biological replicates was calculated for both wildtype and mutant strains and t-test was conducted at $p \leq 0.05$ significance level.

2.2.10   Growth of wheat plants and spore inoculation

Wheat near isogenic lines (NILs) 260-2 (FHB- resistant, *fhb1+*) and 260-4 (FHB-susceptible, *fhb1-*) were used in this study for infection by the fungus. NIL 260-2 differs from 260-4 in bearing the FHB resistant allele (*fhb1+*) at *Qfhb1* locus (Pumphrey et al. 2007). The plants were grown in greenhouse under a 16/8 h light/dark cycle and 20/16°C day/night temperature supplied with cool, white fluorescent lamps. The plants were watered every other day and fertilized every two weeks.

Three inoculation treatments, the wildtype, the mutant, and the empty vector, were done for disease aggressiveness evaluation. The inoculation was performed during anthesis (nearly 7 weeks after planting). Two adjacent spikelets per spike were marked with a sharpie pen on the outer surface of the glumes. The glumes were slightly pulled back and 5 µL of 100,000 spores/mL was injected between the glumes with a pipette in the two adjacent florets. After inoculation, the *F. graminearum* challenged spikes were immediately covered with plastic zip bags supplied with moist cotton balls into them for

72 h to ensure proper humidity and temperature facilitating disease establishment (Li and Yen 2008). The number of diseased kernels were counted and recorded in 7, 14, 21, and 28 days post inoculation (dpi) and the mean percentage of Fusarium damaged kernels (FDK) was calculated for each time point.

For gene expression analysis, 10 µL of 500,000 spores/mL of either wildtype or *ΔfgsiR34+* spores were inoculated in two adjacent florets per spike and covered with plastic zip bags supplied with moist cotton balls for 72 h. The two inoculated spikelets were collected 9 dpi in a 2 mL centrifuge tube. For each treatment, three biological repeats were harvested and the harvested samples were immediately snap frozen in liquid nitrogen and stored at -80°C until RNA extraction was done.

2.2.11 RNA extraction from culture, cDNA synthesis, and Quantitative RT-PCR

Total RNA was extracted from one-week old fungal cultures for both the wildtype and *ΔfgsiR34+* strains using TRIzol reagent and PureLink RNA Mini Kit (Ambion) following manufacturer's instructions. On-column DNase treatment was performed using PureLink DNase (Ambion) to remove DNA from the samples prior to RNA elution. The quality of extracted RNA was determined by resolving it in 1% agarose gel electrophoresis to ensure its integrity and quantified using Nano-Drop ND-1000 Spectrophotometer.

For each sample, one µg of total RNA was used in a 20 µL reaction for cDNA synthesis with GoScript Reverse Transcription System (Promega) and 3' adaptor. An initial incubation of RNA-primer mix was performed at 70°C for 5 min to denature RNA secondary structure and immediately chilled on ice until reverse transcriptase mix was added. Reverse transcription was carried out in a PCR machine with annealing

temperature of 25°C for 5 min, extension at 42°C for 60 min, and inactivation of reverse

transcriptase at 70°C for 15 min. A 5X dilution of the synthesized cDNA was made with

nuclease free water (Promega).

Quantitative RT-PCR (RT-qPCR) was conducted in Applied Biosystems (ABI)

7900HT Real-Time PCR system. For this purpose, a 20 µL reaction was prepared using

2X PowerUp SYBR Green Master Mix (Thermo Scientific), 0.5 µL of 10 µM each of

forward and reverse primers, and 2 µL of the diluted cDNA. The PCR program was set as

UDG activation step at 50°C for 2 min, an initial denaturation of 95°C for 2 min, 40

cycles of 95°C for 15 s and 60°C for 1 min, and the instrument's default dissociation

stage of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Fungal actin gene was used as

an internal control to normalize the Cycle threshold (Ct) value. For each sample, three

technical repeats were conducted and the fold changes were calculated using $2^{-\Delta\Delta Ct}$

method (Livak and Schmittgen 2001). To test the significance of change in expression of

targets between wildtype and mutant strains, student's t-test was conducted at $p \leq 0.05$

level.

2.2.12 Poly(A) tailing coupled with reverse transcription, and RT-qPCR

To determine the expression of siRNA, *fgsiR34*, in culture, a poly(A) tailing

based RT-qPCR was conducted following the protocol described by Shi et al. (Shi et al.

2012). A 10 µL reaction mix was made with total RNA, 3' adaptor, and components from

TaqMan Reverse Transcription System (Applied Biosystems) and poly(A) Tailing Kit

(Ambion). One µL 10 X RT buffer, 2.5 µL 25mM MgCl2, 2 µL 10mM (2.5mM each)

dNTPs, 0.2 µL RNase inhibitor and 0.625 µL MultiScribe reverse transcriptase (from

TaqMan Reverse Transcription System), 0.25 µL 10 µM ATP and 0.5 µL *E. coli* poly(A)

polymerase (E-PAP) (from Ambion) were mixed together in a PCR tube with 200 ng of total RNA and 25 pmol of 3' adaptor. The assembled reaction mix was incubated at 37°C for 1 h and was diluted 10 times with nuclease free water.

For RT-qPCR, the reaction was prepared by adding 10 µL 2X SYBR Green Master Mix, 5 pmol each of forward and reverse primers, 1.5 µL of the diluted cDNA, and nuclease free water to a volume of 20 µL. Each PCR reaction was set up with three technical repeats and placed in ABI 7900HT System with the conditions set up as UDG activation step at 50°C for 2 min, preheating at 95°C for 10 min, forty cycles at 95°C for 15 s and 60°C for 1 min, and the instrument's default melt curve analysis setting of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. For concentration normalization *F. gramiearum* actin gene was used as reference and the fold changes were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The significance of change in expression of *fgsiR34* between wildtype and mutant strains was conducted by *t*-statistic at p≤0.05 level.

2.2.13 RNA extraction from inoculated spikelets, cDNA synthesis, and RT-qPCR

Total RNA was extracted from the inoculated spikelets using TRIzol reagent and PureLink RNA Mini Kit (Ambion). DNase treatment was performed on-column using PureLink DNase (Ambion). The integrity of RNA was determined by resolving it in 1% agarose gel electrophoresis and quantified using Nano-Drop ND-1000 Spectrophotometer.

GoScript Reverse Transcription System with oligo(dT)15 primer (Promega) was used for cDNA synthesis following manufacturer's instructions. Three µg of total RNA from each biological replicate was used for cDNA synthesis and a 20 µL reaction was carried out in a thermocycler with an annealing at 25°C for 5 min, extension at 42°C for

60 min, and inactivation of reverse transcriptase at 70°C for 15 min. A two-fold dilution of the cDNA was made.

Reaction for RT-qPCR was prepared by mixing 10 µL of 2X PowerUp SYBR Green Master Mix (Thermo Scientific), 0.5 µL of 10 µM each of forward and reverse primers, 2 µL of diluted cDNA, and nuclease free water to a volume of 20 µL. Three independent biological replicates were used for gene expression analysis with three technical repeats per biological replicate. Both *Tri5* and *Tri6* genes expression analysis were performed and fungal actin gene was used as the reference for concentration normalization of the Ct values. The relative gene expression was analyzed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). To study the significance of change in expression of targets between the wildtype and the mutant inoculated NILs 260-2 and 260-4, student's *t*-statistic was conducted at p≤0.05 level.

2.2.14 DNA extraction from inoculated spikelets and quantitative PCR assay to determine *F. graminearum* biomass

Genomic DNA was extracted from inoculated wheat spikelets as well as from *F. graminearum* mycelia using QIA DNeasy Plant Minikit (Qiagen) following manufacturer's instructions. The concentration of DNA was measured using Nano-Drop ND-1000 Spectrophotometer.

For quantitative real-time PCR (qPCR) assay of DNA samples extracted from the inoculated spikelets, 2X PowerUp SYBR Green Master Mix, 50 ng of genomic DNA per reaction, and primers specific to *Tri6* gene were all assembled together and a 20 µL reaction volume was set up. For each treatment, two biological replicates with three technical repeats per replicate were used. The Ct averages obtained were then used to

estimate the number of *F. graminearum* cells in the inoculated spikelets by plugging them against the standard curve. The significance of the test was conducted using student's *t*-statistic at p≤0.05.

The foundation of this estimation is that each *F. graminearum* cell has only one copy of *Tri6* gene. Therefore, the copy number of *Tri6* in a DNA sample should represent the number of *F. graminearum* cells in that sample. The illustration below shows the method to calculate *F. graminearum* biomass in each reaction (Yun et al. 2006):

$$M = n \times 1.09 \times 10^{21} \text{g/bp}$$

Where, M = mass per cell; n = genomic size.

Since $n = 3.6 \times 10^7$ bp for *F. graminearum* (Cuomo et al. 2007),

$$M = 3.6 \times 10^7 \text{ bp} \times 1.09 \times 10^{21} \text{g/bp} = 0.039 \text{ picograms}$$

Thus, each *F. graminearum* cell has 0.039 pg DNA, and 10 ng of *F. graminearum* DNA has 256,410 cells.

Using this formula, a standard curve of *F. graminearum* cell number against Ct value of *Tri6* in qPCR assay was generated by making a 10-fold serial dilution of *F. graminearum* DNA using 10 ng of the fungal DNA as the starting material. Primers specific to *Tri6* gene of *F. graminearum* was used to establish the standard curve.

Table 2.1 List of oligonucleotides used in this section of study

Name	Sequence
*fgsiR34- Nco*I F	TTCCATGGCGCCCGGGTGACATCATAGACAGTGGACTAT
*fgsiR34- Asc*I R	TCGGCGCGCCATCAAATCTTGTTAACCGTGA
*fgsiR34- Hind*III F	CGAAGCTTGGAGCTCCTGACATCATAGACAGTGGACTAT
*fgsiR34- Not*I R	GAGCGGCCGCTTCAAATCTTGTTAACCGTGA
3' adaptor	ATTCTAGAGGCCGAGGCGGCCGACATG(T)30(ACG)N
3' adaptor primer	ATTCTAGAGGCCGAGGCGGC
pTMH F	TGCTACATCCATACTCCATCCTTCCC
pTMH R	AGCTGACATCGACACCAACGATCT
GFP spacer F	TCAAAGATGACGGGAACTAC
GFP spacer R	ATCCATCCTCAATGTTGTGT
pUCATPH_*hph* F	GATGTAGGAGGGCGTGGATA
pUCATPH_*hph* R	TCAAGACCAATGCGGAGCA
fgsiR34 expression F	TCAGTAGACAGTGGACTATCTT
Tri4 F	CGGATTCTTGGGTCTTACCA
Tri4 R	GCGGTCCTGAATCTCATCTC
Tri5 F	TCTATGGCCCAAGGACCTGTTTGA
Tri5 R	TGACCCAAACCATCCAGTTCTCCA
Tri6 F	TTGCTTCGACTTTGACTTCG
Tri6 R	CGCTTGAAGTGTTGTCGGTA
Tri10 F	AAGGCTACCGACAGACGAGA
Tri10 R	GCCTCTCCAACAATTGAAGC
Tri14 F	GTTGGAACTCCCGTTGTGAT

Tri14 R	GGGCAAGAATCCCAAGAACT
Fg actin F	ATGGTGTCACTCACGTTGTCC
Fg actin R	CAGTGGTGGAGAAGGTGTAACC
Cutinase F	GTGCAATGTGGACAACAAGG
Cutinase R	TTCCAGAGCCATGTGTGGTA
Exopolygalacturonase F	CACGAGCACCGGAAGATAAT
Exopolygalacturonase R	AGCCTTTCCAATGACGAATG
Pectin esterase F	ATGCCGACAGCGGATACTAC
Pectin esterase R	TCTTCTCGAACCAGGCAGAT
Pectolyase F	GCCACTCTTTCCAACGTCAT
Pectolyase R	CACCGTTGATGAAGGATGTG
Rhamnose F	CCAACAACCAGTGGGAGACT
Rhamnose F	TTTGGTTACCGAGCTTCTGG
Xylanase F	TCGACGTTGCCTACACTGAG
Xylanase R	CCGAGATTCCCCAGACAGTA

2.3   Results

2.3.1   Production of *fgsiR34*-overexpressing mutant (*ΔfgsiR34+*)

Transformation of the fungal protoplasts with *p*TMH44.2-IRT-pUhyg yielded one transformant, *ΔfgsiR34+*. Transformation of the empty vector construct also yielded only one transformant (Figure 2.3). These results indicate that transformation efficiency in *F. graminearum* is quite low. The *ΔfgsiR34+* transformant was able to grow in V8 medium amended with 150 µg/mL hygromycin B while the wildtype fungus was not (Figure 2.4).
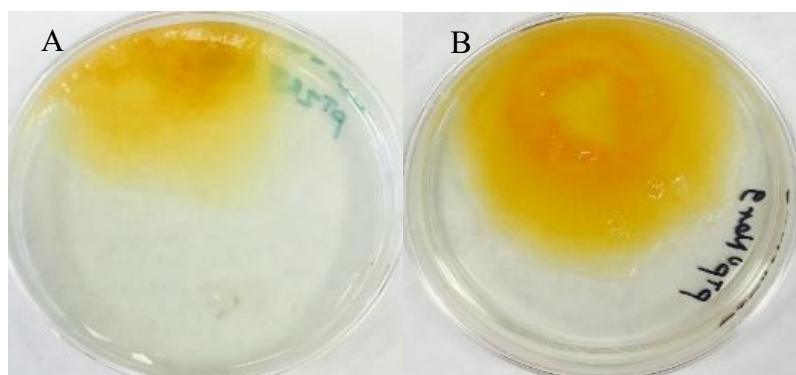
Figure 2.3 Photos showing growth of *ΔfgsiR34+* (A) and empty vector (B) transformants of *F. graminearum* in regeneration medium containing150 µg/mL hygromycin B.
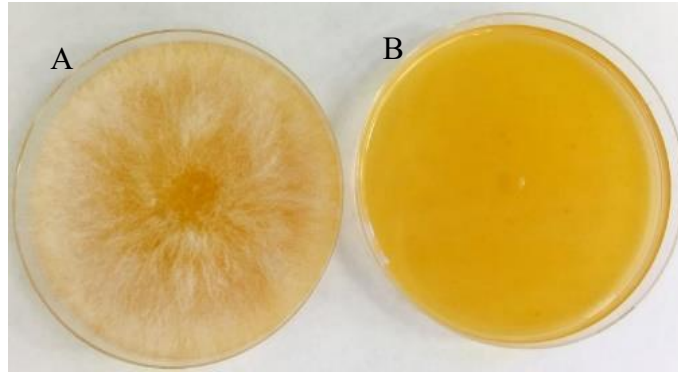
Figure 2.4 Photos showing growth of *ΔfgsiR34+* (A) while no growth of wildtype (B) in V8 medium containing 150 µg/mL hygromycin B

The *ΔfgsiR34+* and the empty vector transformants were confirmed by the PCR amplification of a 280-bp *gfp* spacer and a 794-bp hygromycin phosphotransferase (*hph*) gene from both the transformants (Figure 2.5). In the PCR assay, vectors *p*TMH44.2 and *p*UCATPH were used as positive controls for *gfp* spacer and *hph* gene, respectively. The wildtype *Fg*4 strain was used as a negative control and undoubtedly did not show any amplification for both the genes.

gfp amplification | *hph* gene amplification
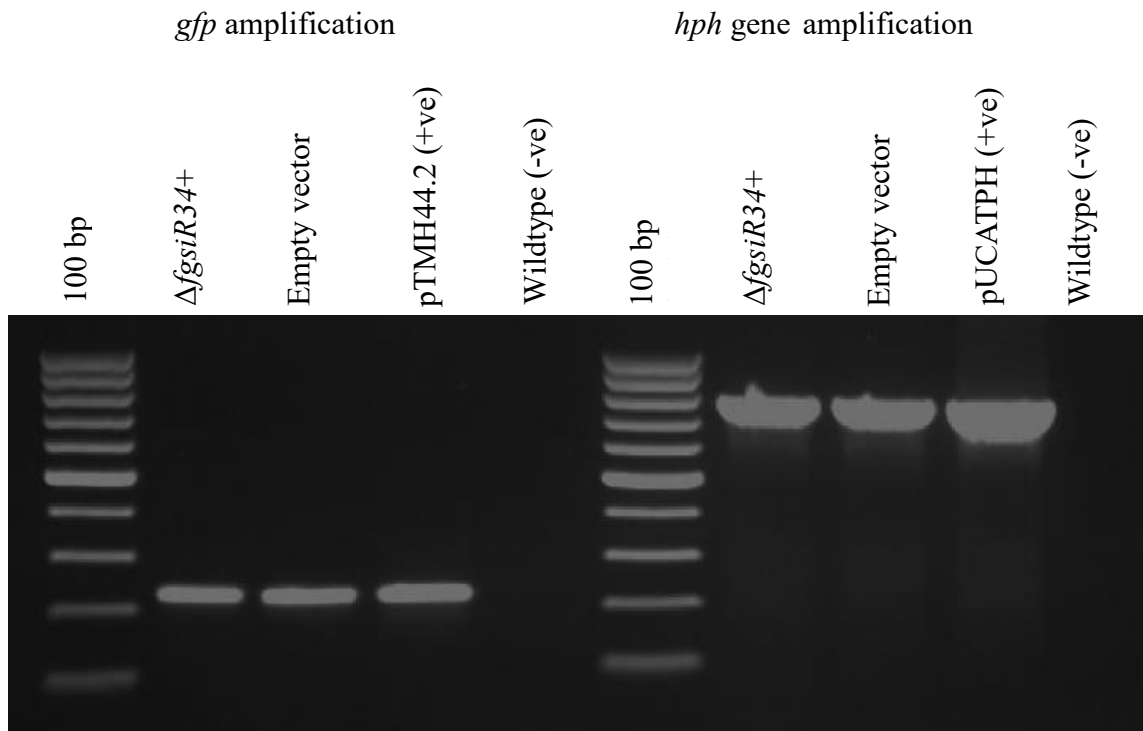
Figure 2.5 Gel image showing amplification of the 280-bp gfp spacer and a 794-bp *hph* fragment in the transformants

2.3.2 *F. graminearum* strains display common morphology/physiology

Macroscopically, all the three strains of *F. graminearum*, wildtype, *ΔfgsiR34+*, and empty vector transformants, exhibit similar morphology. Four biological replicates of each strain were examined for their rates of spore production in liquid medium after 6 d incubation in CMC medium. This assay revealed no significant difference in spore production rates among the strains. Figure 2.6 shows the assay in bar diagram with standard error.

F. graminearum spore production assay
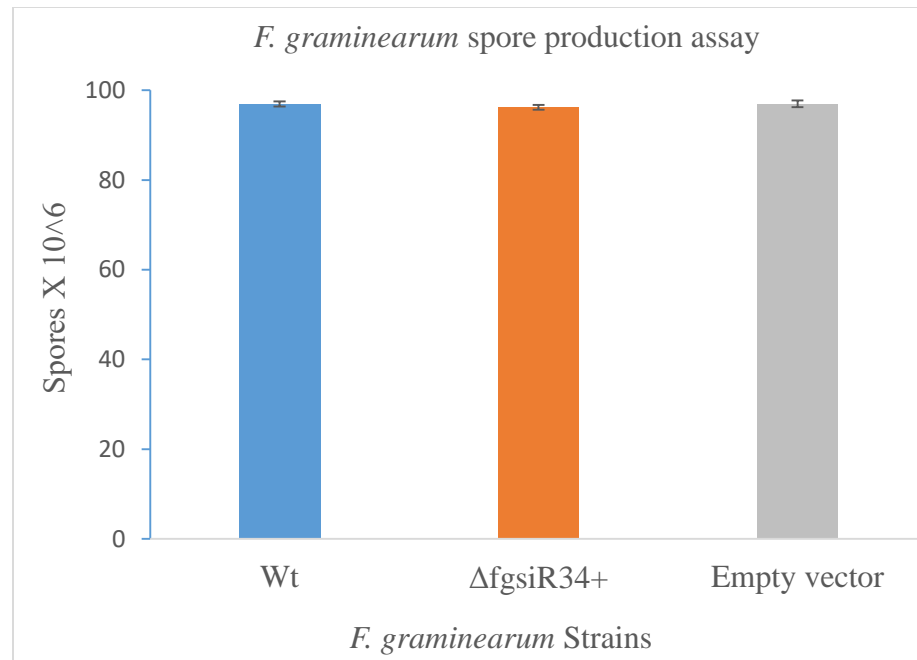
Figure 2.6 Results of spore production assay. *F. graminearum* strains were analyzed for spore production after a 6-day incubation in CMC

Similarly, assay for radial growth was performed to determine whether *fgsiR34* plays an important role in the growth of the fungus. The wildtype and the *ΔfgsiR34+* strains were cultured on four PDA plates side by side. After 5 d of incubation at 28^0C, average diameter of growth for the wildtype and the mutant were found to be 46.25 mm and 45.5 mm respectively. No significant difference in growth between the two strains was observed (Figure 2.7).
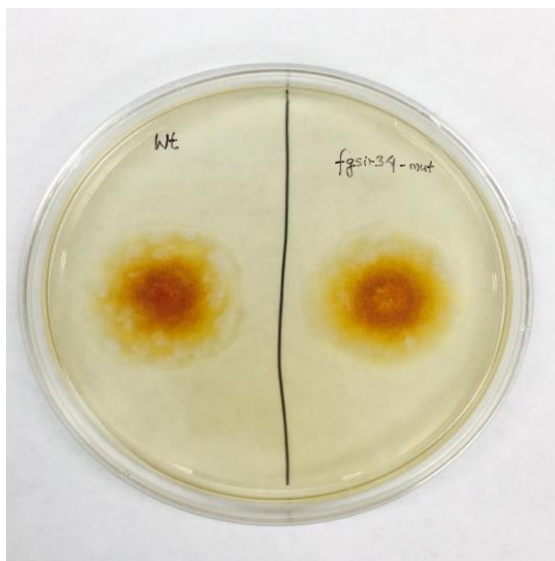
Figure 2.7 Photo showing radial growth
assay of the wildtype and the *ΔfgsiR34+*
strains after 5 d incubation in PDA

2.3.3 Over-expressing *fgsiR34* downregulates *Tri* genes biosynthesis

Poly(A) tailed RT-qPCR assay of the wildtype and the *ΔfgsiR34+* strains revealed

a significant up-regulation of *fgsiR34* in the *ΔfgsiR34+* strain compared to the wildtype.

Our data indicated that *fgsiR34* was upregulated by 3.6 folds in the mutant compared to

the wildtype (Figure 2.8), indicating that the over-expression was successful.

We then investigated relative expression levels of the *Tri4*, *Tri5*, *Tri6*, *Tri10*, and

Tri14 genes between the wildtype and the *ΔfgsiR34+* strains in culture by RT-qPCR

assay. Our results showed that all the five *Tri* genes were significantly downregulated in

the mutant (Figure 2.8). Comparatively, *Tri5*, the gene that encodes trichodiene synthase

that catalyzes the very first step of trichothecene biosynthesis, was significantly

downregulated by nearly six folds. Similarly, the expression of the transcription regulator

genes *Tri6* and *Tri10* were down-regulated by 1.5 and 5.1 folds respectively. *Tri4* which

encodes P450 monooxygenase and catalyzes the first oxygenation step in trichothecene biosynthesis was downregulated by 1.7 folds. Likewise, the expression of *Tri14* gene, which encodes trichodiene oxygenase, was significantly downregulated by 3.4 folds (Figure 2.8).
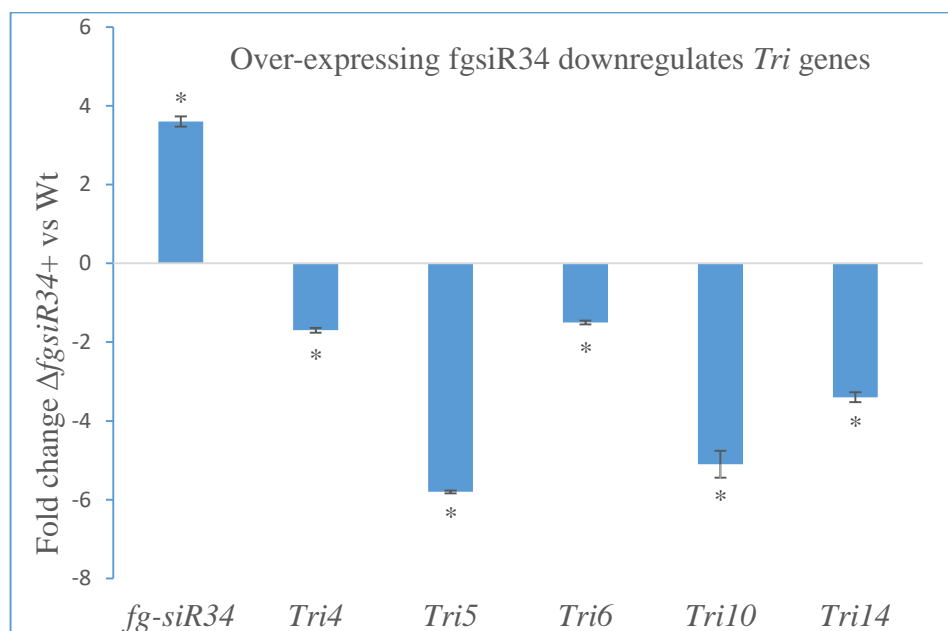
Figure 2.8 Expression pattern of *fgsiR34* and *Tri* genes in the *ΔfgsiR34+* strain compared to the wildtype strain assayed with RT-qPCR. The error bars represent the standard error. The significance level was measured at p≤0.05 (*).

2.3.4 Over-expressing *fgsiR34* upregulates most of the cell wall degrading enzymes

A variety of cell wall degrading enzymes (CWDEs) are produced by the fungus that may assist in FHB pathogenesis by facilitating penetration of the host tissue during the infection. The expression of some important CWDEs were compared between the wildtype and the *ΔfgsiR34+* strains in culture by qPCR. Our results showed that all the CWDEs, but exopolygalacturonase, were significantly upregulated in the mutant (Figure 2.9). Cutinase was the most highly upregulated CWDE (~5 fold up-regulation). Pectin

esterase, pectolyase, rhamnose, and xylanase were all upregulated by almost 2 folds, while exopolygalacturonase was down-regulated by 1.5 folds in the *ΔfgsiR34+* compared to the wildtype.
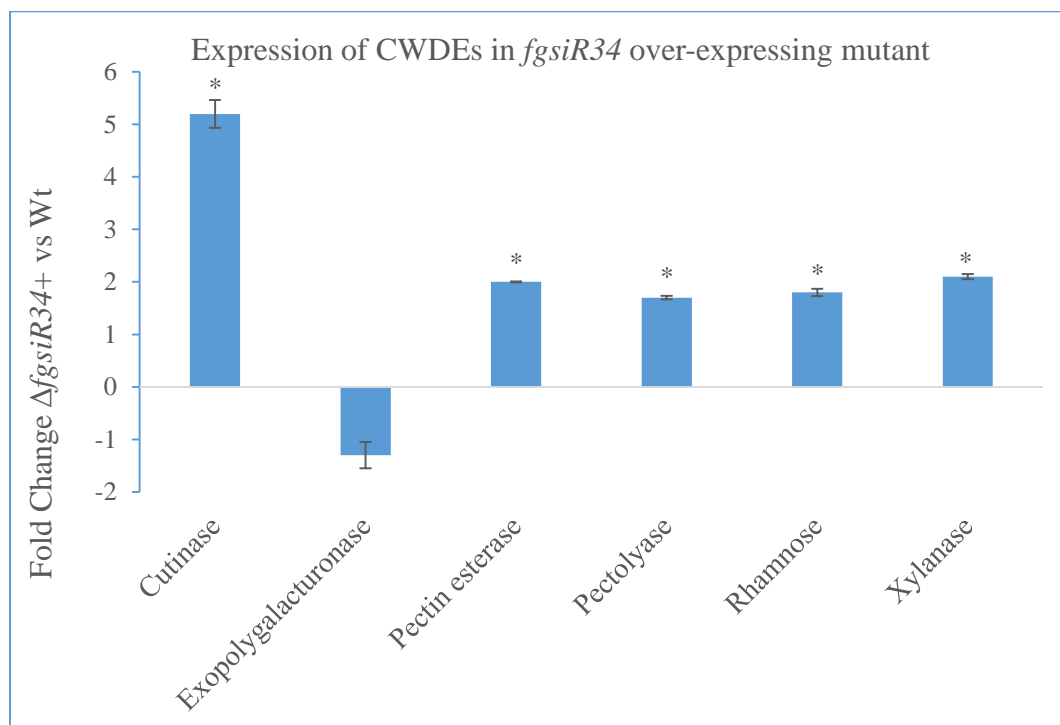
Figure 2.9 Impacts of over-expressing *fgsiR34* in the expression of the genes encoding cell wall degrading enzyme (CWDEs) in culture assayed with RT-qPCR. The error bars show the standard error. The significance level was measured at p≤0.05 (*).

2.3.5 *Tri5* and *Tri6* genes are downregulated in *ΔfgsiR34+* inoculated spikelets

Tri5 and *Tri6* genes were assayed with qPCR for their *in planta* expression in NILs 260-2 and 260-4. The NIL pair were inoculated in the adjacent spikelets with either the wildtype or the *ΔfgsiR34+* spores and the spikelets were harvested 9 dpi. The expression levels of both *Tri5* and *Tri6* genes were significantly downregulated in the *ΔfgsiR34+* inoculated NIL pair compared to the wildtype inoculated NIL pair. The

expression of *Tri5* gene was downregulated by around 14 folds and 8.3 folds in the

mutant inoculated NILs 260-2 and 260-4, respectively (Figure 2.10). Similarly, *Tri6* gene

expression was down by nearly 9 folds in the mutant inoculated NIL 260-2 and almost 6

folds in NIL 260-4 (Figure 2.11).
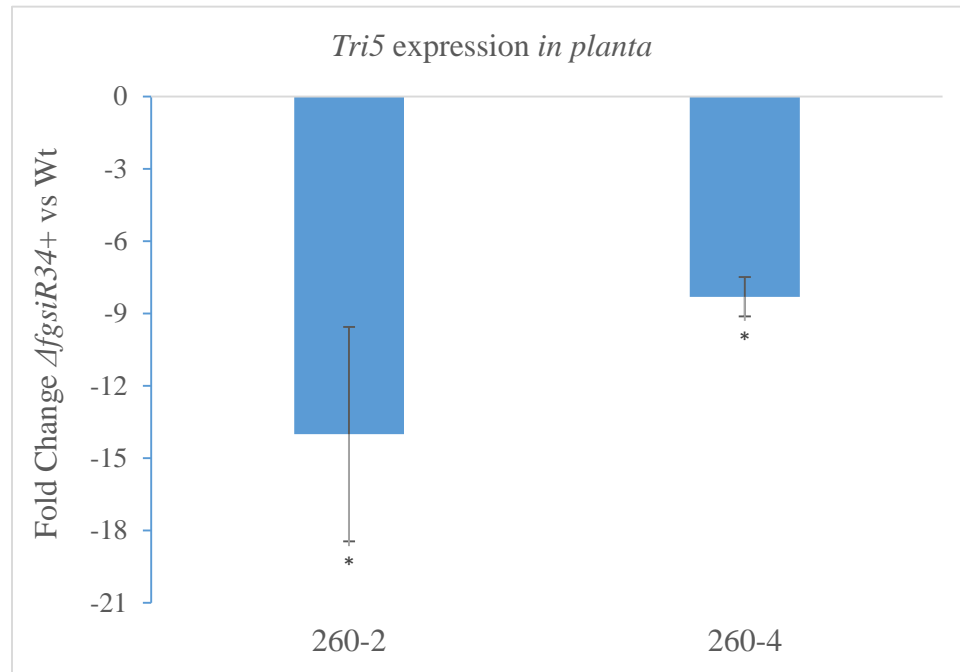
Figure 2.10 *Tri5* expression changes in NILs 260-2 and 260-4 between the *ΔfgsiR34+* and the wildtype strains assayed with RT-qPCR. The error bars show the standard error. The significance level was measured at p≤0.05 (*).
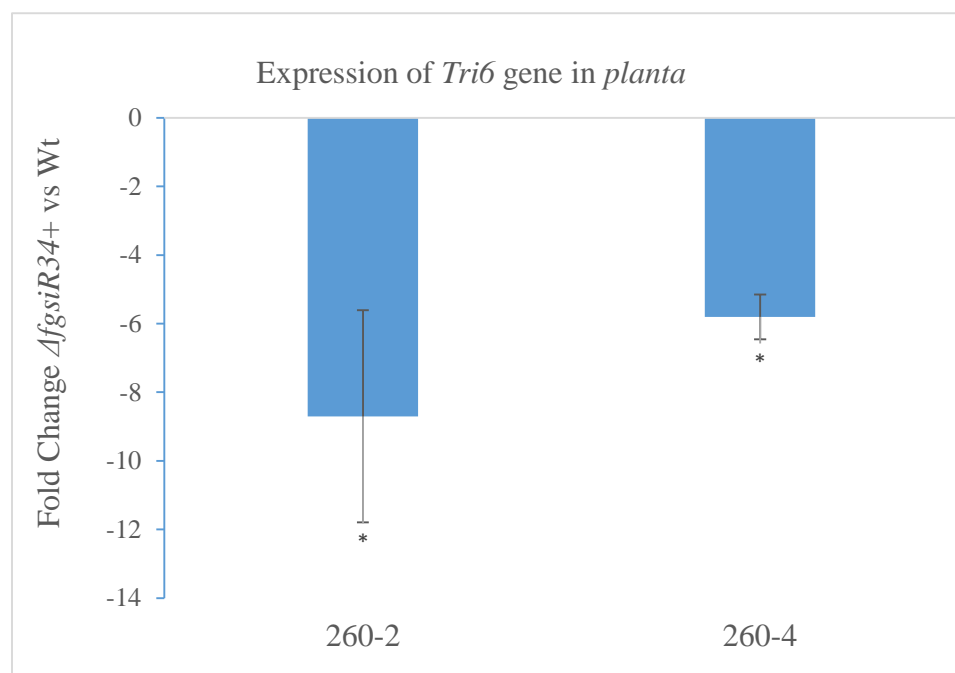
Figure 2.11 Expression changes of *Tri6* in NILs 260-2 and 260-4 between the *ΔfgsiR34+* and the wildtype strains assayed with RT-qPCR. The error bars show the standard error. The significance level was measured at p≤0.05 (*).

2.3.6 Quantifying fungal biomass in inoculated spikelets

The number of *F. graminearum* cells in the inoculated spikelets was estimated by copy number of *Tri6* gene in 50 ng of genomic DNA isolated from the spikelets of the NILs inoculated with the wildtype or the *ΔfgsiR34+* pathogen strain. As indicated in Figure 2.12, the wildtype *F. graminearum* strain has significantly higher biomass in the inoculated NIL 260-4 spikelets than in the inoculated NIL 260-2 spikelets nine days after the inoculation, while similar biomass of the mutant strain was estimated in both of the NILs.
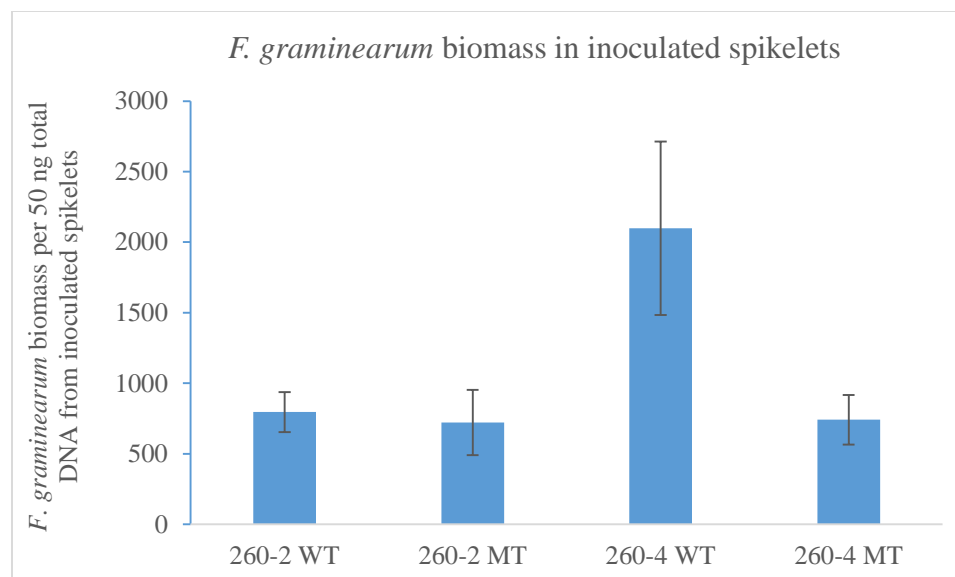
Figure 2.12 Estimation of *F. graminearum* cell numbers in the spikelets of wheat NILs 260-2 and 260-4 infected by the wildtype (WT) or the *ΔfgsiR34+* (MT) fungal spores collected 9 dpi. The error bars represent the standard error.

2.3.7 Disease aggressiveness evaluation

The disease progression was studied in the NIL pair over a period of 7, 14, 21, and 28 days post inoculation with either the wildtype or the *ΔfgsiR34+* fungal spores. Figure 2.13 shows the typical disease phenotypes at each time point in the NILs.
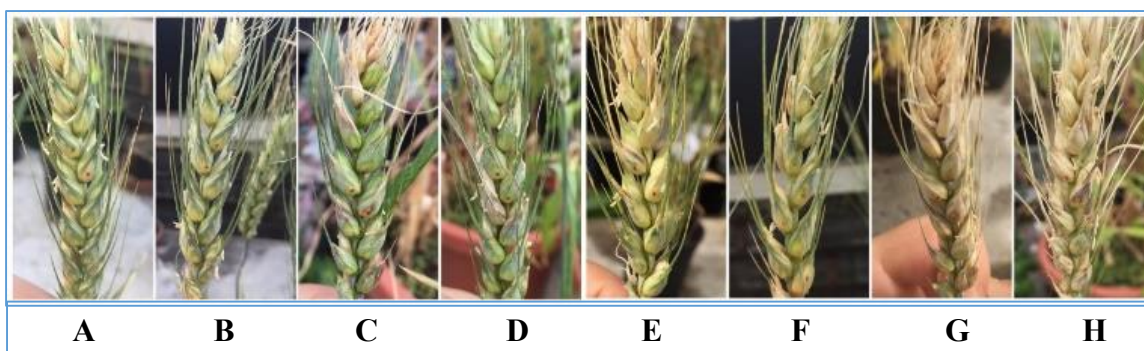
Figure 2.13 Phenotypes of NIL 260-4 spikes after inoculation with either the wildtype or the *ΔfgsiR34+* fungal spores. **A** and **B**: *ΔfgsiR34+*-inoculated spikes 9 dpi, **C** and **D**: *ΔfgsiR34+*-inoculated spikes 21 dpi, **E** and **F**: wildtype-inoculated spikes 9 dpi, **G** and **H**: wildtype-inoculated spikes 21 dpi

Our data suggested that disease progression in the wildtype-inoculated NIL pair was more aggressive than in the mutant-inoculated NILs at every time point we made the observation (Figures 2.14 and 2.15). While the mutant fungal strain showed similar aggressiveness in both NILs, the wildtype strain was almost one fold more aggressive in the FHB-susceptible NIL than in the FHB-resistant NIL at all the time points during the pathogenesis (Figures 2.14 and 2.15).
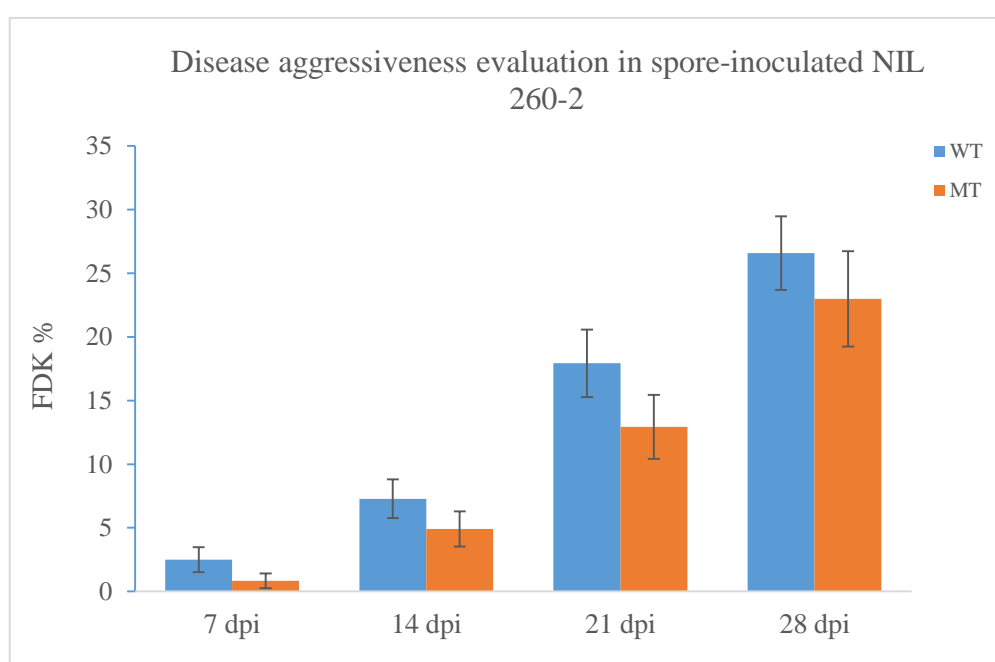
Figure 2.14 Results of disease aggressiveness evaluation of the wildtype-(WT) and the *ΔfgsiR34+* (MT) -inoculated spikes of FHB-resistant wheat NIL 260-2. The error bars show the standard error.
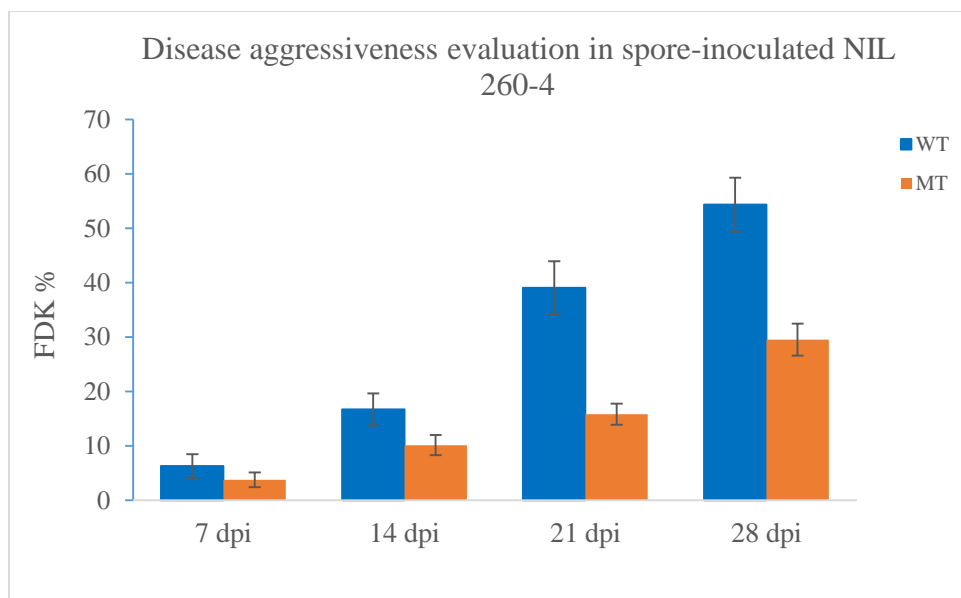
Figure 2.15 Results of disease aggressiveness evaluation of the wildtype (WT)- and *ΔfgsiR34+* (MT)-inoculated spikes of FHB-resistant wheat NIL 260-4. The error bars represent the standard error.

Furthermore, the grain yield (in grams) per spike of wheat was also examined.

The average grain yield per wheat spike was significantly higher in *ΔfgsiR34+* spore-inoculated NILs compared to the wildtype inoculated NILs (Figure 2.16).
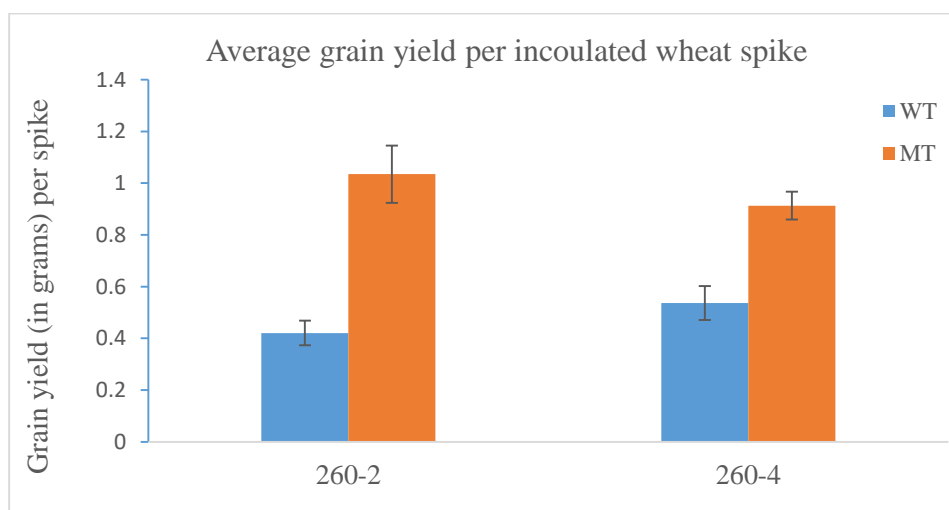
Figure 2.16 Average grain yield (in grams) per wheat spike inoculated with either the wildtype or the *ΔfgsiR34+* spores of *F. graminearum*.

2.4 Discussion

The kingdom fungi consist of a varied group of eukaryotic organisms with diversified roles. Apart from their critical role in maintaining the balance of ecosystem by serving as the primary decomposers, many fungi are pathogenic and pose threats to humans, animals, and plants causing them a wide variety of diseases. The filamentous fungus, *F. graminearum*, is one of the most important pathogen of small cereal grains causing severe losses in crop yield and marketability worldwide. Therefore, it is crucial to understand the mechanisms underlying the pathogenesis of this fungus in cereal grains.

With the discovery of a conserved eukaryotic gene silencing mechanism in 1998 (Fire et al. 1998), the genetic dissection of a number of eukaryotes, including fungi, has revealed diverse sRNAs and their biogenesis pathways. Studies conducted in *Saccharomyces pombe*, *Neurospora crassa*, and several other fungi have suggested that RNAi pathways in fungi function in gene regulation, genomic defense, and heterochromatin formation (Dang et al. 2011). In the filamentous fungus, *N. crassa*, several classes of sRNAs have been identified, including qiRNAs (sRNAs that interact with AGO protein QDE-2, Quelling Deficient-2) (Lee et al. 2009), miRNA-like RNAs (milRNAs), and dicer-independent siRNAs (disiRNAs) (Lee et al. 2010). These findings have broadened our understanding of sRNA biogenesis and RNAi pathways in filamentous fungi, to which *F. graminearum* belongs to. It has been observed that RNAi in filamentous fungi mostly engenders in posttranscriptional gene silencing rather than transcriptional gene silencing (Freitag et al. 2004; Chicas et al. 2005).

In the present study, we investigated the possible role of silencing RNA *fgsiR34* in FHB pathogenesis of wheat by generating an *fgsiR34* over-expressing mutant.

Previously, to study the possible role of sRNAs in FHB pathogenesis, *DCL-2* gene, which

plays an important role in sRNA biogenesis, was knocked down from the wildtype *F.*

graminearum isolate 4 and *Δdcl2-* mutant was generated. Of the 52 sRNAs identified,

only one siRNA, *fgsiR34*, was significantly downregulated in the *Δdcl2-* mutant. This

result implied that *fgsiR34* was the only siRNA that was controlled by *DCL-2* gene in *F.*

graminearum. The *Δdcl2-* mutant was observed to be less pathogenic *in planta* compared

to the wildtype. The DON biosynthetic genes were also significantly downregulated and

DON production was significantly reduced in the *Δdcl2-* mutant inoculated wheat

spikelets compared to the spikelets inoculated with the wildtype fungal spores. All these

results suggested an important role of silencing RNA *fgsiR34* in regulating DON

biosynthesis and hence FHB pathogenesis (Galla 2014).

To further elucidate the role of the silencing RNA *fgsiR34* we generated *fgsiR34*

over-expressing mutant (*ΔfgsiR34+*) in the present study using IRT method with slight

modification (McDonald et al. 2005). The transformation of the wildtype fungal

protoplasts with the construct containing both sense and anti-sense strands of *fgsiR34*

(*p*TMH44.2-*fgsiR34* IRT-*p*Uhyg) yielded only one transformant. The presence of inverse

repeat in the transformant was confirmed by the amplification of a 280-bp GFP spacer in

the mutant, which forms the loop of the IRT (Figure 2.5).

The spore production rate in liquid medium as well as growth rate in solid

medium was studied and compared between the wildtype and the mutant strains. No

significant difference was observed among wildtype, *ΔfgsiR34+*, and empty vector strains

for spore production rate in liquid medium. The radial growth assay on solid medium

further confirmed that wildtype and *ΔfgsiR34+* behave morphologically similar in culture

conditions and *fgsiR34* has no significant role in maintaining the growth and physiology of the fungus.

We studied and compared the expression levels of *Tri* genes and several CWDEs, between the wildtype and the *ΔfgsiR34+* strains in culture. Over-expressing *fgsiR34* significantly downregulated the expression of *Tri4*, *Tri5*, *Tri6*, *Tri10*, and *Tri14* (Figure 2.8). Since *Tri* genes encode the enzymes that catalyze the production of DON (Figure 1.4), our data suggested that *fgsiR34* negatively regulates DON biosynthesis pathway. DON is the final product of trichothecene biosynthesis pathway and is considered as one of the most important and economically significant trichothecene mycotoxin produced by the fungus (Proctor et al. 2009). The first step in trichothecene biosynthesis is catalyzed by trichodiene synthase which is encoded by *Tri5* gene (Hohn and Beremand 1989) (Figure 1.4). Therefore, majority of studies have focused on *Tri5* gene to understand *Fusarium*-wheat interaction. In our study, among the *Tri* genes that we investigated, *Tri5* was the one that showed the most altered expression level in the *ΔfgsiR34+* strain relative to the wildtype strain in culture (Figure 2.8). It was significantly downregulated by almost 6 folds in the mutant. NCBI hit blast shows that at 997 bp upstream of *Tri5* gene, the sequence of *fgsiR34* has a 60% match (Appendix 7). The seed region of *fgsiR34* (2-11 bases) matches from the 918th to the 927th bases in chromosome 2 of *F. graminearum* genome. Therefore, it could be possible that the incorporated or over-expressed *fg*-siRNAs in the *ΔfgsiR34+* strain might be negatively regulating *Tri5* expression by targeting and degrading mRNA with complementary base pairing using RNAi mechanism. However, the transcription factor regulators *Tri6* and *Tri10* genes were also significantly downregulated in the mutant relative to the wildtype. Since these two *Tri*

genes are the global gene regulators (Seong et al. 2009), their downregulation might have influenced the downregulation of other *Tri* genes including *Tri5* as well.

All the CWDEs that were studied were significantly upregulated in the *ΔfgsiR34+* strain, except exopolygalacturonase which was downregulated but not significant (Figure 2.9). The gene that encodes the enzyme cutinase was upregulated by more than five folds. The genes that encode for the enzymes pectin esterase, pectolyase, rhamnose, and xylanase were upregulated by around two folds. This could be to compensate for the decreased pathogenicity and reduced *Tri* genes biosynthesis in the mutant. Our study suggested that *fgsiR34* positively regulates the genes that encode for CWDEs. However, the upregulation of CWDEs in the *ΔfgsiR34+* strain was not sufficient to help disease progression in the wheat inoculated with these mutant spores (Figures 2.13 to 2.15).

Previous studies on CWDEs have not been able to provide conclusive results on their significance in fungal pathogenicity (Annis and Goodwin 1997). There have been reports suggesting that mutants lacking in expression of a specific CWDE do not necessarily have decreased virulence (Bowen et al. 1995; Wu et al. 1997) because disruption of a certain enzyme may be compensated by secretion of other isoforms manifesting similar properties (Hamer and Holden 1997). A study conducted by Sella et al reported that when *xyr1*, the gene that encodes for the enzyme xylanase that is responsible for degrading xylan of the host cell wall, was disrupted in *F. graminearum*, the virulence of *Δxyr1* mutant seemed to be unaffected in the inoculated wheat spikelets and soybean seedlings compared to the wildtype strain (Sella et al. 2015). Though CWDEs function to degrade plant cell walls and penetrate the tissue (An et al. 2005), studies have suggested that trichothecenes are the major factors responsible for the

progress or spread of infection on both wheat and maize rather than CWDEs (Proctor et al. 1995a; Harris et al. 1999).

We also examined the expression levels of two important *Tri* genes, *Tri5* and *Tri6*, in *F. graminearum*-inoculated FHB-susceptible or FHB-resistant wheat cultivars. Our study showed that both *Tri5* and *Tri6* genes were significantly downregulated in the spikelets of both the susceptible and the resistant wheat cultivars inoculated by *ΔfgsiR34+* compared to the wildtype-inoculated spikelets of the cultivars (Figures 2.10 and 2.11). This result was in accordance with the reduced *Tri* gene biosynthesis in culture (Figure 2.8). Previous studies have shown that mutants with disrupted or deficient *Tri5* gene were greatly reduced in virulence and toxin production in inoculated wheat spikelets compared to those inoculated by the wildtype strains (Proctor et al. 1995a; Desjardins et al. 1997). A similar study demonstrated that disease spread did not occur in wheat spikes inoculated with *Tri5*-disrupted mutants though initial infection was observed to be established (Bai et al. 2002).

From our disease aggressiveness evaluation, it was observed that the disease progression was more severe in the wildtype-inoculated NIL 260-4 compared to the mutant-inoculated one (Figure 2.15). In fact, when the biomass of *F. gramineaum* in the inoculated NIL pair was analyzed by the qPCR assay of the DNA isolated from the inoculated spikelets, there was no significant difference in the biomass between the susceptible and resistance NILs inoculated with *ΔfgsiR34+* fungal spores (Figure 2.12). Contrastingly, the wildtype inoculated NIL 260-4 was observed to have significantly higher biomass of *F. graminearum* than the rest of the treatments (Figure 2.12).

We observed that *ΔfgsiR34+*-inoculated FHB-susceptible cultivar had similar disease aggressiveness as the *ΔfgsiR34+*-inoculated FHB-resistant cultivar (Figure 2.15). This result was consistent with the average grain yield per spike in the *ΔfgsiR34+* inoculated NIL pair, which showed no significant difference between the average grain yield in the *ΔfgsiR34+* inoculated NIL 260-2 and 260-4 (Figure 2.16). However, this siRNA does not seem to have a significant phenotypic effect in the FHB-resistant NIL 260-2 (Figure 2.14). Clearly the pathogen interacts with the susceptible and the resistant NILs differently.

In conclusion, our study shows that *fgsiR34* negatively regulates DON biosynthesis pathway and positively regulates the genes encoding CWDEs. This is in contrast to our previous finding in the *Δdcl2-* mutant where knocking down *DCL-2* downregulated the expression of *fgsiR34* and also suppressed the *Tri* genes expression and DON production in the *Δdcl2-* inoculated spikelets (Galla 2014). Though both of our studies signify the important role of *fgsiR34* in DON biosynthesis pathway, more elucidation is required to fully understand the mechanism. Performing Southern blotting analysis would be helpful to reveal the IRT integration pattern in the transformants. RNA-seq analysis to study differentially expressed genes in the pathogen strains as well as the inoculated wheat spikelets might be helpful to functionally characterize potential pathogenicity factors. The interaction of the pathogen with the host is probably a counterbalance expression of different genes, CWDEs, hormones, metabolites, or other factors yet to be unraveled. A better understanding of all these mechanisms will be valuable for the proper elucidation of the molecular interactions between *F. graminearum* and its host and therefore manipulating effective control strategy.

Chapter 3 Exploring the mechanisms by which *fgsiR34* functions in FHB pathogenicity

3.1 Introduction

Fungi are the most important group of plant pathogens causing severe diseases in crops with serious yield losses worldwide. *Fusarium graminearum* is an ascomycetous fungal pathogen that causes Fusarium head blight (FHB) disease in wheat and other small cereal grains. The disease is favored mostly in warm and humid conditions during anthesis and early stages of kernel development (Gilbert and Tekauz 2000). FHB has a devastating effect on cereal grains with severe yield losses. In the United States alone, from 1991 to 1997, FHB outbreaks in wheat and barley have caused an estimated direct loss of about $1.3 billion (Johnson et al. 2003). The disease not only results in direct yield losses through low-weight kernels and reduced grain yield but also causes indirect loss in grain quality by accumulation of potent trichothecene mycotoxins, mainly deoxynivalenol (DON) (Bai et al. 2001; Dexter et al. 2003).

DON is considered as the most important mycotoxin produced by *F. graminearum,* and acts as a virulence factor for the establishment and spread of the disease in wheat (Proctor et al. 1995a; Desjardins and Hohn 1997; Bai et al. 2002; Jansen et al. 2005; Ilgen et al. 2008). DON resists heat and is stable during storage and processing of harvested grains (Wolf-Hall et al. 1999). Grains contaminated with DON are unsafe for human and animal consumption causing vomiting in humans and reduced feed intake and weight loss in animals (Desjardins and Hohn 1997). Since the role of DON as a virulence factor in wheat has been reported in a number of studies (Proctor et al. 1995a; Desjardins et al. 1996; Bai et al. 2002; Jansen et al. 2005; Maier et al. 2006;

Cuzick et al. 2008), understanding mechanisms that regulate toxin production is essential

for the effective FHB management and control strategies.

The biosynthesis of DON is catalyzed by several enzymes that are encoded by *Tri*

genes located on three different chromosomes in *F. graminearum* (Kimura et al. 1998b;

Brown et al. 2001; Jurgenson et al. 2002; Kimura et al. 2003a; Meek et al. 2003;

Alexander et al. 2004). The core cluster is located in chromosome 2 and consists of 12

genes (Lee et al. 2008), including *Tri5*, which encodes the enzyme trichodiene synthase

that catalyzes the first step of DON biosynthesis (Hohn and Vanmiddlesworth 1986;

Hohn and Desjardins 1992; Desjardins et al. 1993). The regulatory genes, *Tri6* and *Tri10*

are also located in the same cluster (Proctor et al. 1995b; Tag et al. 2001; Peplow et al.

2003b). The two other gene loci consists of a two-gene mini cluster *Tri1* and *Tri16* on

chromosome 1 (Brown et al. 2003; Meek et al. 2003) and a single gene *Tri101* on

chromosome 4 (Gale et al. 2005; Alexander et al. 2009).

DNA methylation is the most thoroughly studied epigenetic modification of

chromatin that can cause inheritable modification of genetic information. It is often

associated with suppression of gene expression (Law and Jacobsen 2010; Castel and

Martienssen 2013; Matzke et al. 2015). In most eukaryotic organisms, there is an addition

of a methyl moiety to cytosine residues that are located 5' to guanine in the CpG

dinucleotide (Holliday and Grigg 1993). This epigenetic modification most notably

influences gene expression in CpG islands, which are CpG-rich areas located in the

promoter regions of many genes (Bird 1985; Bird 1992). CpG islands are stretches of

DNA spanning more than 200 bases, with at least 50% G+C content and a ratio of an

observed to statistically significant CpG frequency of at least 0.6 (Portela and Esteller

2010). Methylation of the cytosine residue in the CpG islands is known to be a gene silencing mark that transcriptionally represses genes. DNA methylation has important regulatory effects on gene expression, silencing of repetitive and centromeric sequences from fungi to mammals, plant epigenetic variation, imprinting, X-chromosome inactivation, cancers, and other diseases (Jullien and Berger 2010; Koh and Rao 2013; Schmitz et al. 2013; Smith and Meissner 2013). There have been reports of aberrant methylation of normally unmethylated CpG islands in transformed and aberrant cells (Antequera et al. 1990). It is therefore crucial to determine the exact position of the modified bases.

The enzymatic pathway that leads to the methylation of DNA involves multiple DNA methyltransferases (DNMTs) enzymes. In eukaryotic systems, at least three functional DNMTs have been identified. DNMT1 is the primary enzyme responsible for maintaining methylation pattern, preferentially by adding methyl groups to hemi-methylated DNA during cellular replication (Robertson 2001). Other functional methyl transferases DNMT3a and DNMT3b contribute to *de novo* DNA methylation activity (Okano et al. 1999; Rottach et al. 2009), while another methyl transferase DNMT2 does not appear to have significant activity of DNA methylation (Robertson 2001). Methylated cytosine (5-methyl cytosine or 5mc), at promoter regions, is often associated with the binding of methyl-CpG associated domain-containing proteins, such as MeCP2. The translocator proteins at this region can result in the oxidation of 5-methyl cytosine (5mc) to 5-hydroxymethyl cytosine (5hmc), preventing access to DNMTs and thereby maintaining an unmethylated state of the promoter region, and therefore leading to

transcriptional activation. Similar to cytosine methylation, cytosine hydroxymethylation is also involved in epigenetic regulation (Branco et al. 2012).

In our previous studies, to investigate the possible role of small RNAs (sRNAs) in DON biosynthesis pathway, an RNAi-induced knockdown mutant, *Δdcl2-*, of *Dicer-like 2* (*DCL-2*) gene was generated from the wildtype *F. graminearum* isolate 4, *Fg4* (Galla 2014). *DCL-2* is a key player in sRNA biogenesis. It cleaves a long double stranded RNA into short 21-25 nt sRNA duplexes. These sRNAs are exploited by RNAi machinery to target and degrade cognate mRNA molecules leading to post transcriptional gene silencing (Elbashir et al. 2001; Hammond et al. 2001). Our study in *Δdcl2-* mutant suggested that of 52 sRNAs that were identified and functionally validated in *Fg4*, only single silencing RNA, *fgsiR34,* was significantly downregulated compared to the wildtype. The expression of *Tri* genes, *Tri4*, *Tri5*, *Tri6*, *Tri10*, and *Tri14*, were all significantly down-regulated in *Δdcl2-*. The disease development in FHB-susceptible wheat was reduced by ten folds in *Δdcl2-* compared to the wildtype. NCBI blast hit showed that this silencing RNA has a potential target site upstream of *Tri5* gene (Appendix 6). This finding led us to propose that *fgsiR34* might be affecting regulation of *Tri5* gene by methylation and hence the regulation of trichothecene biosynthesis pathway.

In this study we are detecting methylation status of the seed region of silencing RNA *fgsiR34* by bisulfite treatment of genomic DNA and methylation-specific PCR (MSP). At 997 bp upstream of *Tri5* gene there is a 60 percent match of *fgsiR34* sequence and 100 percent match in the seed region of *fgsiR34* on *F. graminearum* chromosome 2 (Appendix 7).

3.2   Materials and Methods

We used two *F. graminearum* strains in this study- *Fg*4 as the wildtype and an *fgsiR34* over-expression mutant (*ΔfgsiR34+*) of *Fg*4. *Fg*4 is a local isolate used in our lab for *F. graminearum* pathogenicity study. The *ΔfgsiR34+* was generated from the wildtype by over-expressing silencing RNA, *fgsiR34*, using Inverse Repeat Transgene (IRT) method (McDonald et al. 2005) with slight modification. In our previous study, we investigated the expression of *Tri* genes in the wildtype and the mutant and observed a significant downregulation of all the *Tri* genes in the mutant compared to the wildtype. *Tri5* gene, the key player of trichothecene biosynthesis, was down-regulated by almost six folds. All these results made us to hypothesize that seed region of *fgsiR34* upstream of *Tri5* gene might be methylated and therefore, affecting the regulation of *Tri5* by DNA methylation.

3.2.1   Culture of *F. graminearum*

The wildtype and the *ΔfgsiR34+* strains used in this study were cultured in potato dextrose agar (PDA) for fungal growth. Ten µL of fungal spores of the wildtype and the mutant type were inoculated separately on each PDA plate. After inoculation the plates were kept in dark and incubated at 28°C for 7 d.

3.2.2   Genomic DNA extraction

Genomic DNA was extracted from both the fungal cultures after a week using Qiagen's DNeasy Plant Mini Kit. The quality of the extracted DNA was tested by resolving in 1% agarose gel to assure its integrity and quantified using Nano-Drop ND-1000 Spectrophotometer.

3.2.3 Bisulfite modification of DNA

The EpiTect Bisulfite Kit from Qiagen was used for sodium bisulfite conversion

of DNA. One µg of genomic DNA was used as the starting material to which 85 µL of

dissolved sodium bisulfite was added in a PCR reaction tube. Sodium bisulfite provides

optimal pH for complete conversion of unmethylated cytosines to uracils. Thirty-five µL

of DNA Protect Buffer was added to the mix which helps in preventing the fragmentation

of DNA usually associated with bisulfite treatment at high temperature and low pH

values. The volume of the mixture was adjusted to 140 µL by adding RNase free water

prior to the addition of dissolved bisulfite mix and DNA protect buffer. The PCR tubes

containing the bisulfite reaction mix were briefly spun to bring the contents to the bottom

of the tube. The bisulfite DNA conversion was performed using a thermal cycler. The

thermal cycler condition for bisulfite conversion is shown in Table 3.1:

Table 3.1 Thermal cycler conditions for bisulfite conversion

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min	60°C
Denaturation	5 min	95°C
Incubation	175 min	60°C
Hold	Indefinite	20°C

3.2.4   Purification of modified DNA

After the bisulfite conversion reaction was complete, the bisulfite treated DNA was cleaned up and purified using the EpiTect spin columns (Qiagen) following the manufacturer's protocol. The concentration of treated purified DNA was measured using the Nano-Drop ND-1000 Spectrophotometer and the DNA was stored at -20°C in aliquots for MSP.

3.2.5   Methylation-specific PCR

Two sets of primer pairs were designed to the gene of interest that annealed to sequences containing CpG dinucleotides. One primer pair specific for the sequence recognized methylated CpGs (M primer), whereas the other primer pair specific for the sequence recognized unmethylated CpGs (U primer). The primer pairs specific for methylated and unmethylated sequences were designed for the region nearly 100 bases upstream of *Tri5* gene on chromosome 2. The PCR reactions for two primer sets were performed on separate tubes to amplify the target the gene. A third primer pair specific to the sequence upstream of *Tri5* region was also designed for bisulfite unmodified DNA to check the efficiency of bisulfite modification. This primer pair was designated as W (unmodified or wildtype sequence) primer set and it amplified genomic DNA unmodified by bisulfite treatment.

All the reactions for PCR were set up with 10 µL 2X GoTaq Green Master Mix (Promega), 1 µL of template DNA, 1 µL of each 10 µM forward and reverse primers and nuclease free water to a final volume of 20 µL. The thermal cycler program was set at an initial denaturation temperature of 95°C for 2 min, forty cycles at 95°C for 45 s, 55°C for 45 s, 72°C for 60 s, a final extension of 72°C for 5 min, and an infinite hold at 4°C. The

PCR products were then resolved on 1% agarose gel side by side for comparison. The gel was visualized using ABS Imaging System.

3.2.6 Cloning and sequencing of target DNA

The amplified product obtained for the specific primer pair was purified using the QIAGEN PCR Purification kit. The purified PCR product was ligated into the pGEM-T vector (Promega) using 2X Rapid ligation buffer and T4 DNA ligase, transformed into competent *E. coli* cells (JM 109), and plated onto LB/Amp/IPTG/X-Gal plates. The plates were incubated overnight at 37ºC. The transformants were analyzed by colony PCR to confirm the presence of inserts. An isolated white colony was picked and grown in 2 mL of LB broth containing 100 µg/mL of ampicillin overnight at 37ºC with shaking at 220 rpm. Plasmid DNA was isolated and purified using the PureLink™ HQ Mini Plasmid Purification Kit and sent for sequencing by GenScript.

3.2.7 RNA extraction, cDNA synthesis, and RT-qPCR of the non-coding RNA transcript from the *fgsiR34* seed region of *F. graminearum* genome

To further investigate the potential role of *fgsiR34* on its seed region between *Tri6* and *Tri5* genes, expression of non-coding RNA transcript between the wildtype and
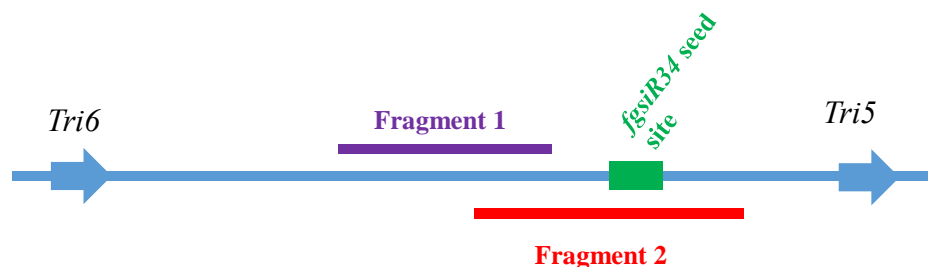
Figure 3.1 Illustration (not in scale) showing the regions of transcript that were amplified by RT-qPCR assay.

ΔfgsiR34+ strains was studied with RT-qPCR. Two sets of primers were designed: one set flanking upstream of the *fgsiR34* seed region and the second set that amplified the seed region as shown in Figure 3.1.

First of all, total RNA was extracted from one-week-old fungal cultures for both the strains using TRIzol reagent and PureLink RNA Mini Kit (Ambion) following manufacturer's instructions. To remove DNA from the samples prior to RNA elution, on-column DNase treatment was performed using PureLink DNase (Ambion). The quality of extracted RNA was determined by resolving it in 1% agarose gel electrophoresis to ensure its integrity and quantified using Nano-Drop ND-1000 Spectrophotometer.

For each sample, one µg of total RNA was used in a 20 µL reaction for cDNA synthesis with GoScript Reverse Transcription System (Promega) and 3' adaptor. Reverse transcription was carried out in a PCR machine with annealing temperature of 25°C for 5 min, extension at 42°C for 60 min, and inactivation of reverse transcriptase at 70°C for 15 min. A 5X dilution of the synthesized cDNA was made with nuclease free water (Promega).

Quantitative RT-PCR (RT-qPCR) was conducted in Applied Biosystems (ABI) 7900HT Real-Time PCR system. For this purpose, a 20 µL reaction was prepared using 2X PowerUp SYBR Green Master Mix (Thermo Scientific), 0.5 µL of 10 µM each of forward and reverse primers, and 2µL of the diluted cDNA. The PCR program was set as UDG activation step at 50°C for 2 min, an initial denaturation of 95°C for 2 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, and the instrument's default dissociation stage of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Fungal actin gene was used as an internal control to normalize the Cycle threshold (Ct) value. For each sample, three

technical repeats were conducted and the fold changes were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). To test the significance of change in expression of targets between wildtype and mutant strains, student's *t*-test was conducted at p≤0.05 level.

Table 3.2 List of primers used in this section of study

Name	Sequences
Unmodified Sequence primer (W) F	GAGGAGCGCAGCATCGCCAGTA
Unmodified Sequence primer (W) R	CATATATCCCGCGGAGTTGGCT
Methylated_seed_Tri5 F	CGATGATAGGTATGTTAGATAATCGT
Methylated_seed_Tri5 R	TCTATACAAAATACAAAATCCCGTT
Unmethylated_seed_Tri5 F	TGTTAGTGATGATAGGTATGTTAGATAATT
Unmethylated_seed_Tri5 R	TCTATACAAAATACAAAATCCCATT
Fragment 1_seed site F	CGGACTTGTCACAGTTGCTG
Fragment 1_seed site R	TCAGGGTGTTCATGGTAGGG
Fragment 2_seed site F	TGACAGGCATGCCAGATAAC
Fragment 2_seed site R	CCGTTTTCAATTAACCCATGTC

3.3   Results

3.3.1   Amplification of unmodified genomic DNA

W primer set amplified genomic DNA unmodified by bisulfite treatment as shown in Figure 3.2. No amplification was seen for bisulfite modified DNA with W primer set indicating that the bisulfite treatment of the DNA sequence was complete and that there was no technical flaw during the treatment procedure.
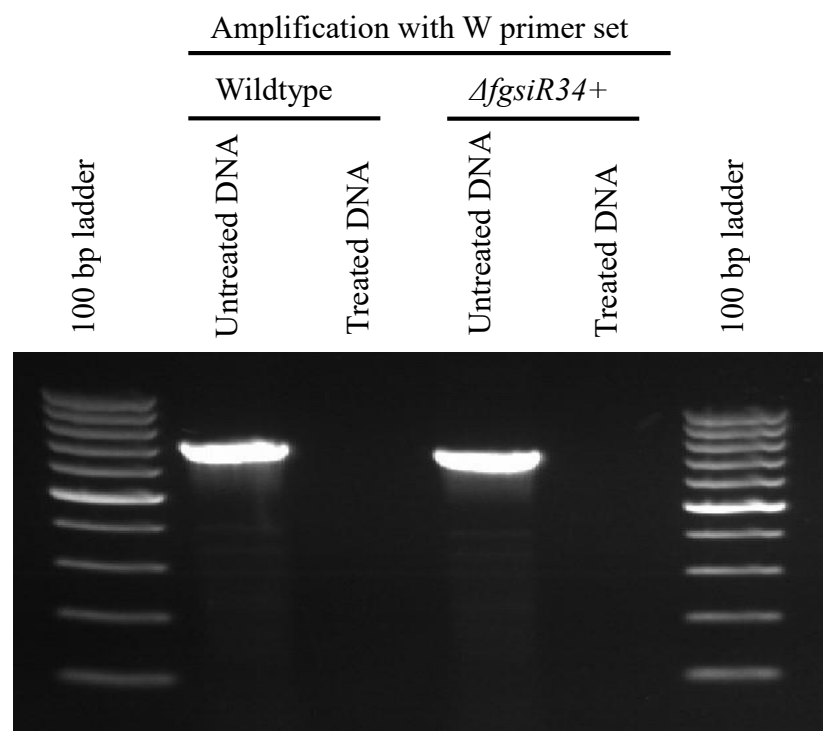
Figure 3.2 A gel image showing amplification of bisulfite unmodified DNA of *F. gramineaum* with W primer set.

3.3.2   No differential methylation observed between *F. graminearum* strains

The methylation pattern of the seed region of *fgsiR34* (nearly 1000 bp upstream of *Tri5* gene) was studied between the wildtype and the mutant strains to find out the possibility whether *fgsiR34* regulates *Tri5* gene by methylation (Appendix 7). As shown

in Figure 3.3, a 264 bp amplification was seen only for unmethylated primer set for both

the wildtype and the mutant strains indicating that the region was unmethylated. No band

was seen for the methylated primer set. The sequencing result also verified that the

sequence on the seed region was unmethylated (Appendix 7). This study ruled out the

possibility of suppression of *Tri5* gene expression in the mutant strain due to DNA

methylation and suggested that some other mechanisms might be involved.
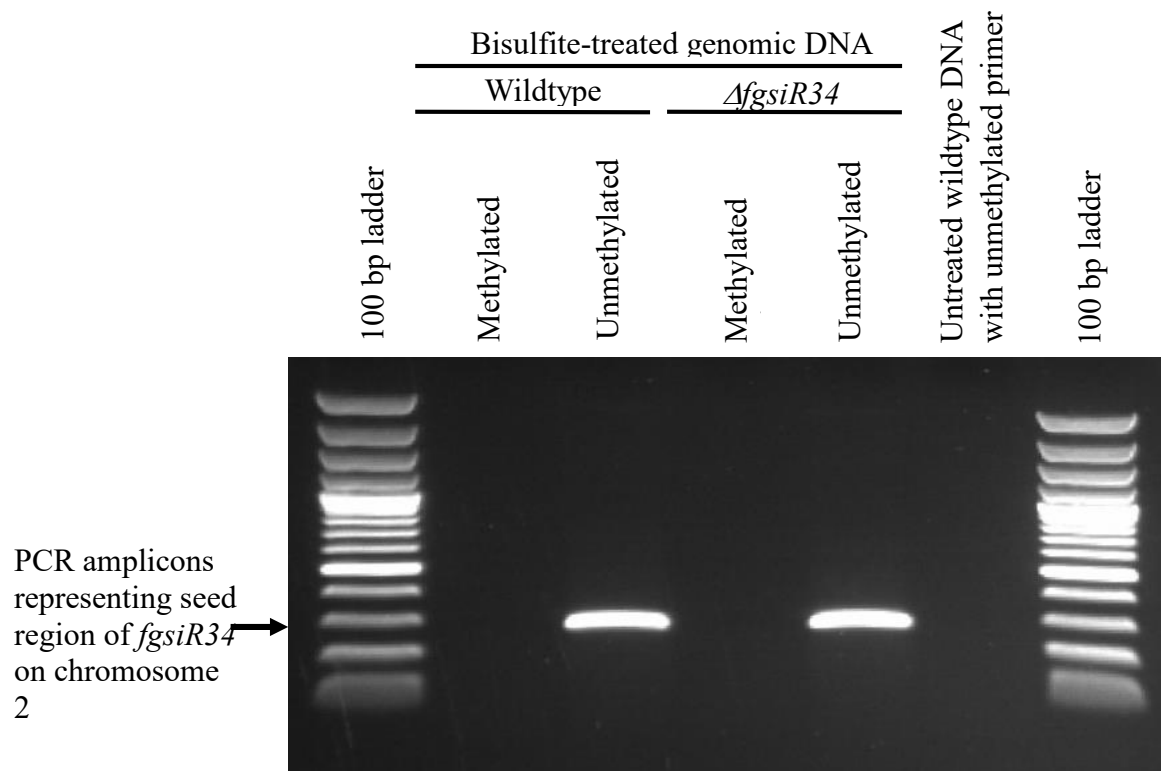
Figure 3.3 Amplification of seed region of *fgsiR34* upstream of *Tri5* gene after bisulfite
treatment of genomic DNA of wildtype and mutant strains using U (unmethylated) primer
set.

3.3.3 Upregulation of non-coding transcript in the *ΔfgsiR34+* relative to the wildtype strain

Using RT-qPCR assay, we studied the presence and the accumulation of the non-coding RNA transcribed from the *F. graminearum* chromosomal region between *Tri6* and *Tri5* that hosts the *fgsiR34* target. As shown in Figure 3.4, our data first demonstrated that non-coding RNA was transcribed from this region. It further showed a significant larger accumulation of this non-coding RNA transcript in the *ΔfgsiR34+* strain compared to the wildtype strain. The first fragment was upregulated by around 2.1 folds while the second fragment was upregulated by around 7.4 folds.
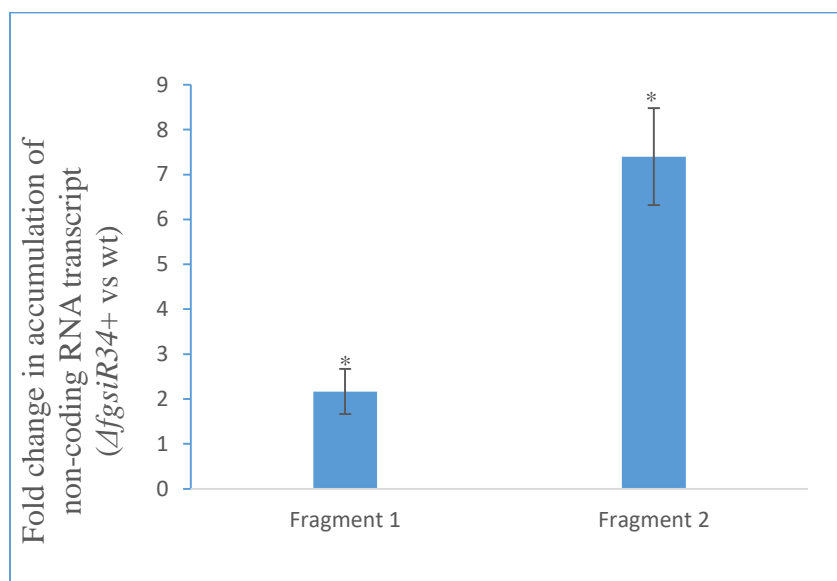
Figure 3.4 Graph showing fold change in accumulation of the non-coding RNA transcripts between the wildtype and the *ΔfgsiR34+* mutant strains. The significance level was measured at $p \leq 0.05$ (*).

3.4 Discussion

In this study, we analyzed the methylation pattern of the seed region of silencing RNA *fgsiR34* at nearly 1000 bp upstream of *Tri5* gene to elucidate if methylation has any role in down-regulating the expression of trichothecene genes, the genes that are involved in mycotoxin biosynthesis in *F. graminearum*. Since DNA methylation suppresses transcription, it plays an important role in gene silencing and inactivation. DNA methylation usually occurs on the fifth carbon of the cytosine residue (5-methyl cytosine), particularly at CG-rich regions of the genome. The presence of methylated cytosine base directly interferes with the binding of transcription factor to DNA, and therefore represses the expression of the corresponding gene (Prendergast and Ziff 1991; Clark et al. 1997). Alternately, methylcytosine-binding proteins may hinder the passage of regulatory elements to the corresponding gene sequence (Yu et al. 2000).

Sodium bisulfite modification of DNA is one of the most important methods to determine the methylation status of individual cytosine residues. When genomic DNA is subjected to sodium bisulfite, unmethylated cytosine residues are deaminated and converted to uracil, while methylated cytosines are unaffected by the bisulfite treatment since the methyl group at their 5-carbon atom prevents deamination. This treatment brings about a change in the DNA sequence depending on the methylation status of individual cytosine base (Frommer et al. 1992). Analyzing bisulfite treated DNA with PCR that uses two specific primer pairs, methylated primer pair that recognizes methylated CG region and unmethylated primer pair that recognizes unmethylated CG region, helps to differentiate pattern of individual cytosine methylation in the gene of interest (Herman et al. 1996).

To study the methylation pattern at the seed sequence upstream of *Tri5* gene, we used two strains of *F. graminearum*- wildtype and Δ*fgsiR34*+. After bisulfite treatment and MSP we could not find a differential methylation for the gene of interest between the two strains. Also we identified the seed sequence of *fgsiR34* to be unmethylated. This led us to depict that there is no role of methylation in repressing the expression of *Tri* genes and some other mechanisms might be involved in this downregulation.

A few studies have been made on DNA methylation in fungi (Antequera et al. 1984; Russel et al. 1985; Abbas et al. 1986; Russell et al. 1987; Buckner et al. 1988; Reyna-Lopez et al. 1997; Martienssen and Colot 2001). There has been a report of differential fungal methylation during morphogenesis in dimorphic fungi (Reyna-Lopez et al. 1997). A study conducted by Antequera et al. (1984) on the incidence and the pattern of cytosine methylation in fungi showed disparate distribution of cytosine methylation ranging from undetectable levels (≤0.1%) to low but detectable levels (~0.2 to 0.5%) (Antequera et al. 1984). Proffitt and his colleagues failed to detect methylated cytosines in the budding yeast *Saccharomyces cerevisiae* (Proffitt et al. 1984).   However, a slime mold, *Physarum polycephalum* was observed to be hyper-methylated (Evans and Evans 1970; Evans et al. 1973; Whittaker and Hardman 1980; Peoples et al. 1985). Moreover, Bull and Wotton reported a heavy methylation of amplified DNA in transformants of the model fungus, *Neurospora crassa*, while the wildtype had very low levels of cytosine methylation in its genome. Their results implicated a mechanism for a significant *de novo* DNA methylation (Bull and Wootton 1984). Because lower eukaryotes, such as fungi and *Drosophila*, lack significant methylation in their respective genomes (Hattman et al. 1978; Urieli-Shoval et al. 1982; Feher et al. 1983; Achwal et al.

1984; Antequera et al. 1984; Proffitt et al. 1984), DNA methylation might not have an extensive role to control gene expression (Doerfler 1983; Bird 1984). Nevertheless, they might be involved in DNA recombination or repair process (Bull and Wootton 1984).

Recent studies have shown that non-coding RNAs play a significant role not only in gene silencing mechanism by degrading the cognate mRNA sequences via RNAi (Fire et al. 1998; Elbashir et al. 2001), but also serve as activators of gene expression by targeting gene regulatory sequences. This novel phenomenon was first discovered by Li et al in 2006 and is referred to as RNA activation (RNAa) (Li et al. 2006; Place et al. 2008; Lopez et al. 2016; Meng et al. 2016). Several small RNAs, including short double stranded RNAs (dsRNAs), small hairpin RNAs (shRNAs), and micro RNAs (miRNAs), have been shown to positively regulate target sequences and are called small activating RNAs (saRNAs) (Li et al. 2006; Place et al. 2008; Turunen et al. 2009; Huang et al. 2012). The target sequences for RNAa in most of the cases are sense and antisense non-coding sequences (Schwartz et al. 2008; Matsui et al. 2010; Matsui et al. 2013). The non-coding RNAs thus activate gene transcription by targeting non-coding regulatory regions in the promoters of the gene (Li et al. 2006).

Our result demonstrated that a non-coding RNA is transcribed out of the region between *Tri6* and *Tri5* on chromosome 2 in *F. graminearum* where *fgsiR34* potentially targets. At this moment, we do not know the exact function of this non-coding transcript. Nor do we know why the two fragments of this non-coding transcript we assayed had a differential accumulation in the *ΔfgsiR34+* mutant. However, the significant larger increase in accumulation of this transcript in the *ΔfgsiR34+* strain than in the wildtype (Figure 3.4) hinted at its possible functional link with *fgsiR34*. Moreover, this might

suggest the existence of multiple non-coding transcripts made from the same region. Further research is needed to get a better picture.

Therefore, we hypothesized that siRNA, *fgsiR34*, might be acting as an saRNA and activating promoter activity of the gene nearby its seed site by binding to or targeting at the antisense transcript, which results in a functional promoter for gene transcription. The significantly higher accumulation of this transcript in the *ΔfgsiR34+* mutant relative to the wildtype and the differential accumulation of different fragments of this transcript in the mutant might suggest a more complex nature of *fgsiR34* in regulating gene expression. Identifying natural targets induced by this siRNA and studying their gene expression profiles might give a clear idea on the regulation mechanism of this siRNA.

Chapter 4 General Conclusions and Future Perspectives

4.1 General conclusions

Fusarium head blight (FHB) is a devastating disease of wheat, and other small cereal grains occurring in all major-cereal growing areas of the world. The disease is so severe that it results in losses of grain yields and quality, and contaminates grains with harmful mycotoxins (McMullen et al. 1997). Globally, *Fusarium graminearum* is the most predominant and problematic species of *Fusarium*. The effective strategy to control FHB is a high priority to assure the safety of harvested grains for food and feed consumption as well as for fermentation purposes (Urban and Hammond-Kosack 2013).

The long-term goal of our research is to advance our understanding of the molecular mechanisms of host-pathogen interaction during FHB of wheat. In particular, we studied the role of silencing RNA, *fgsiR34* in trichothecene biosynthesis pathway. In our previous study, when *DCL-2* gene was knocked down in *F. graminearum* isolate 4 (*Fg*4), *fgsiR34* was the only siRNA that was found to be significantly downregulated in the *Δdcl2-* mutant. At the same time, all *Tri* genes on chromosome 2 that we examined were also significantly downregulated, leading to suppression of DON production in the *Δdcl2-* mutant inoculated FHB susceptible wheat cultivar Y1193-6 (Galla 2014). Therefore, we hypothesized that *fgsiR34* positively regulates the *Tri* gene core on chromosome 2.

To test our hypothesis, we successfully generated an *Fg*4 mutant that overexpressed *fgsiR34* (*ΔfgsiR34+*) and studied it *in culture* and *in planta* with comparison to the wildtype *Fg*4. Our data showed that the *ΔfgsiR34+* mutant generally

behaves similarly as the *Δdcl2-* mutant both *in culture* and *in planta*. Specifically, the *Tri*

genes tested were downregulated in both mutants, the genes encoding CWDEs also

behaved similarly in both the mutants, and both mutants had significantly reduced FHB

pathogenicity and grew poorly in either FHB-resistant or FHB-susceptible wheat

cultivars. These observations suggest that *fgsiR34* was responsible for all of the gene

expression changes that we observed in the *Δdcl2-* mutant, playing a critical role in

regulating *Tri* genes and thus DON biosynthesis in *F. graminearum*. This is not

surprising since the only target site by *fgsiR34* in *F. graminearum* genome lies between

Tri6 and *Tri5* genes on chromosome 2. What surprised us is that our data from the

ΔfgsiR34+ mutant does not support our hypothesis developed from our analysis of the

Δdcl2- mutant but indicates a more complex mechanism of this regulation. Our data

suggest that methylation does not play a role in the *fgsiR34*'s regulation of *Tri* genes and

that the *fgsiR34* target region seems to transcribe non-coding RNAs. More surprisingly,

these non-coding RNAs seemed to differentially respond to *fgsiR34* overexpression.

Therefore, more research is needed to find out if these non-coding RNAs also have

different roles in the regulatory mechanism of *fgsiR34*.

     NCBI BLAST hit shows that the coding sequence of *fgsiR34* is two 391-bp

repeated sequences in the *F. graminearum* mitochondrial genome (Appendix 1).

fgsiR34's coding sequence is also present in the mitochondrial genomes of other

Fusarium spp.: - one in *F. oxysporum*, two in *F. culmorum*, and three in *F. gerlachii*.

There are multiple questions that need to be answered to fully comprehend the role of

fgsiR34 in FHB pathogenesis in wheat. Does *F. graminearum* have a copy of its

mitochondrial genome in its nuclear genome? Otherwise, how does the biogenesis of mitochondrial small RNAs occur? How does the small RNA get into the cytosol?

Further research on the role of *fgsiR34* should expand the knowledge that we currently have. Southern blotting analysis should reveal the patterns of integration of the inverted repeat transgenes (IRTs) in the transformants because the integration patterns also impact the gene expression levels in the mutant strains as demonstrated by Scherm et al. (Scherm et al. 2011). Whole transcriptome analysis of the mutant and the wildtype strains should enable the characterization of all RNA transcripts and reveal the differentially regulated genes in both the strains. Electrophoretic mobility shift assay (EMSA) should reveal the action of *fgsiR34* in the functioning of *Tri6* gene. We could generate an *fgsiR34* knockout mutant using CRISPR (clustered regularly inter-spaced palindromic repeats)/Cas9 genome editing technology. Since we already have knocked down and overexpressed mutants of *fgsiR34*, knockout mutant of *fgsiR34* might help us to conduct neat assays, although knocking out a gene might sometimes be lethal.

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Appendix 1. *fgsiR34*'s coding sequence is in two 391-bp repeated sequences in *F. graminearum* mitochondrial genome

- *fgsiR34* has 22 nucleotides (5'-TCAGTAGACAGTGGACTATCTT-3')

- Its coding sequences are in mitochondrial genomes of several *Fusarium* species- one in *F. oxysporum*, two in *F. graminearum*, two in *F. gerlachii* and three in *F. culmorum*

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13561 caaaataatc gtgaagtagc ttctgctgtg caagcgagtg agcaaccacc aacagtagaa tcccctatgg
13641 tgaagacagc tttgcggatg cgccttatcc atatgaggct gaaagtccaa aggataatga taacaatagt
13721 aaggtcgtac agcagtggct tcgggtgacc aagtggtaga gggggaagag attgctctaa tatccgcaga
13801 agtgaagggg atttggcttt ttcaccgcaa ttttttatac cagagtcaac tccagatagt atttctagcg
13881 tcctattaat acgcaagcta accaggggat gggtgagatc ttagagtcaa atcttgttaa ccgtgatgag
13961 tagatggaga tttgtatcgt gaagttatga ataatgcggt aggggaagcc gtaatggaaa gtataaatcc
14041 cttcctgatc taccaactag taatcctgaa ccggtggata ctgaagatag tccactgtct actgaagtta
14121 tatgatgtca gacagtgaat tcttcgagct agaacgtcaa atcgcgagtc taaagccttc ttacttagaa
14201 gtgaggtaga cggttctggc caagatgcgg ctgacccaga ggctattata atatcttcag atgatagttc
14281 ggtgattatg gtaataatta tagtgaggca agtccgatta cgagcttacc cgggaatagg gctggccaaa
14361 agaggctatt ataagatctt cagatgatag ttctagtgag ggggtaccaa cgccacctaa ggaaacagat
14441 gaccgcttaa tactcctgta gcggaagtta ttcacgatag aactggaact aggttattga ctgttgatat
14521 ttaacggtaa tcggcccacc aaatgcacca gaacctcgtt tatggagtgt gggaaatgca aattattatc
14601 tcctgatttt ttttacgagc attatcctag gggtacggag gtaatgcctc ctaataatta tccgtcccaa
14681 tttatatacc tccgcctatg gataaactac gatgcgaatc tgatgggcgt attggggaaa tttgcatatg
14761 ttcgctactg tagtaccatt agtaaattac cctaggtcga gttatataga aggagcgcta ttcatgaaat
14841 ctcagaacca ttaggagata aggatttgag tgatggtttt aatttgagtg atcgtttgaa tttttcttcg
14921 ctaatgctta ttttgcacca gagccaactc cagatacaga tagtatatct agcgataact ctagtcctat
15001 gctaaccagg aggaggctgg taagccggag gttactctaa tatccgcaga tgatatgacc
15081 ttcaccgcaa ttttttatac cagagtcaac tccagatagt atttctagcg acaactctag tcctattaat
15161 accaggggat gggtgagatc ttagagtcaa atcttgttaa ccgtgatgag ccaatgcaag tagatgaaga
15241 gaagtcatga ataatgtggt aggggaagcc acaatggaaa ctataaatcc tatcctactt cttcctgacc
15321 taatcccgaa ccggtggata ctgaagatag tccactgtct actgaagata gtccactgtc tatgatgtca
15401 tcttcgagct agaacgggaa atatctaagc taggaaagtc tataggaaga gacaagaaag
15481 gggcgcaagc tcttactggt agatagctat aggtattata taagctatct accgtagggg acataattta
15561 tgctgacctt gcatgttaga aggctcggtt cgaatccgag tgtctccata taagcttgtg aagctcaatt
```

Appendix 2. Structure of pAN52-3
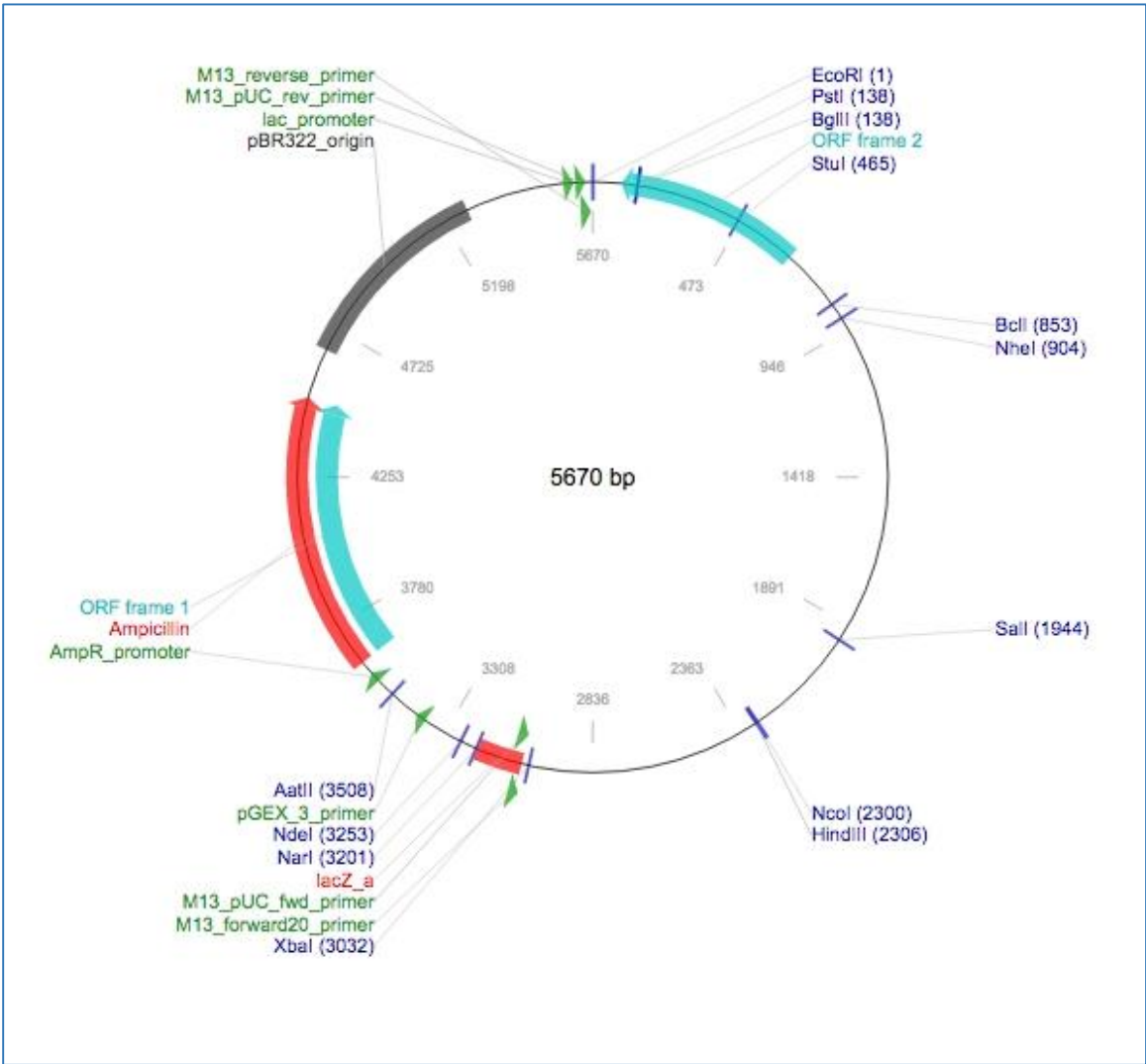
Feature Name	Start	End	Enzyme Name	Cut
M13_forward_primer	3061	3045	EcoRI	1
M13_pUC_fwd_primer	3076	3054	PstI	138
lacZ_a	3054	3197	BgIII	138
pGEX_3_primer	3389	3411	StuI	465
AmpR_promoter	3570	3598	BcII	853
Ampicillin	3640	4500	NheI	904
pBR#@@_origin	4655	5274	SaII	1944
lac_promoter	5583	5612	NcoI	2300
M13_pUC_rev_primer	5626	5648	HindIII	2306
M13_reverse_primer	5647	5665	XbaI	3032
			NarI	3201
			NdeI	3253

ORF	Start	End		AatII	3508
ORF frame 2	659	87			
ORF frame 1	3640	4500			

Appendix 3. Schematic structure of *p*TMH44.2
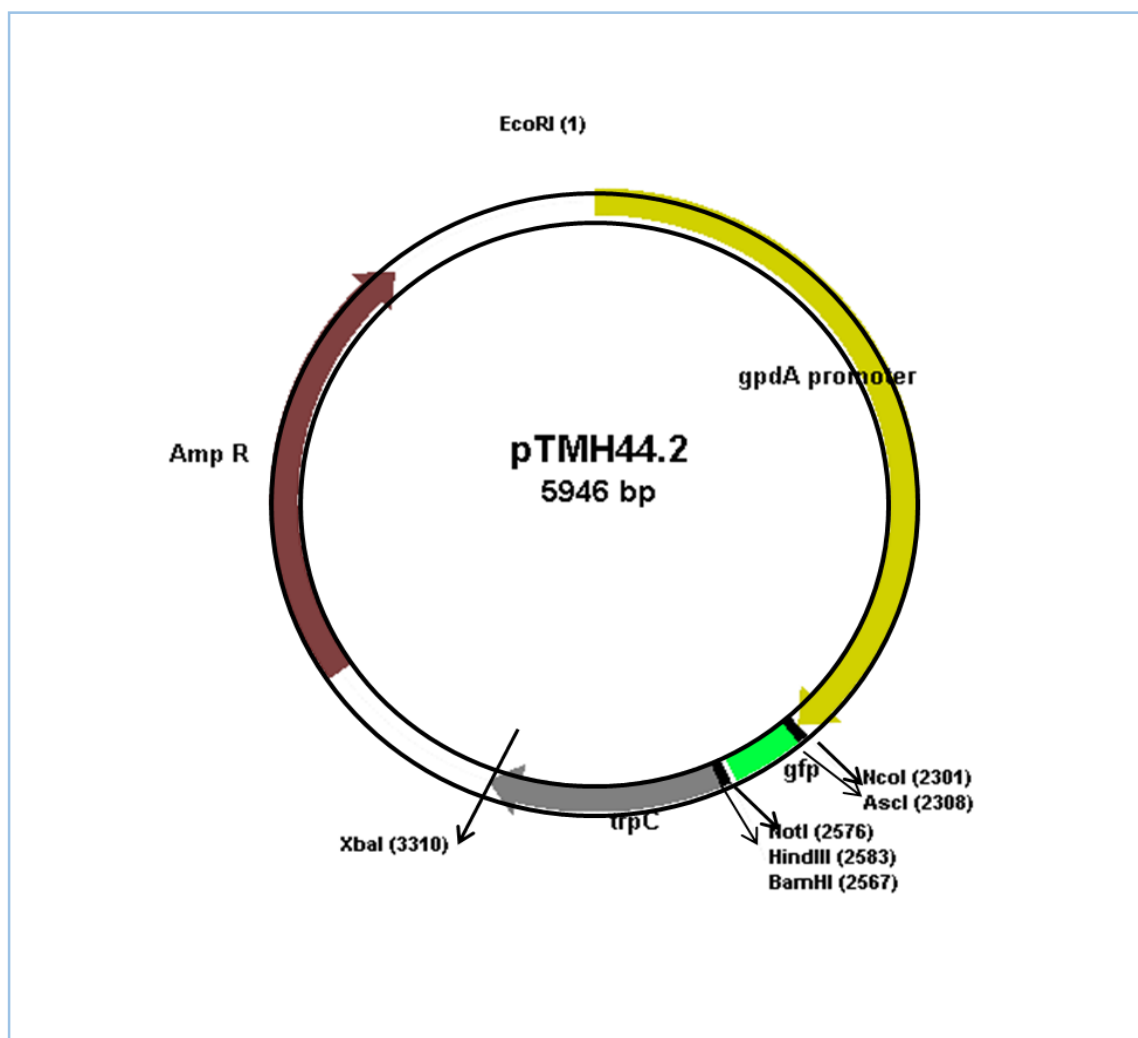
Construction of *p*TMH44.2

 *p*TMH44.2 is a modification of *p*AN52-3 by addition of a 280 bp fragment of *gfp* spacer with a forward primer having an *Nco*I site and nested *Asc*I site and a reverse primer having a *Hind*III, nested *Not*I site, and nested *BamH*I site. This fragment was then ligated into the *Nco*I/*Hind*III sites of *p*AN52-3 between a high expression fungal promoter and a terminator (McDonald et al. 2005).

Appendix 4. Disease aggressiveness evaluation of *F. graminearum* wildtype and *ΔfgsiR34*+ strains

260-2 wildtype	Inoculation date	dpfi				Number of Spikelets	FDK (%)			
		7	14	21	28		7	14	21	28
Sample 1	6/7/2016	0	2	8	8	22	0	9.1	36.36	36.36
Sample 2	6/7/2016	0	4	6	9	25	0	16	24	36
Sample 3	6/7/2016	0	0	4	8	28	0	0	14.29	28.57
Sample 4	6/7/2016	0	0	2	4	26	0	0	7.69	15.39
Sample 5	6/7/2016	2	2	8	13	26	7.7	7.7	30.77	50
Sample 6	6/7/2016	1	2	2	4	27	3.7	7.41	7.41	14.81
Sample 7	6/7/2016	0	0	1	3	23	0	0	4.35	13.04
Sample 8	6/7/2016	0	1	3	5	25	0	4	12	20
Sample 9	6/7/2016	0	3	7	7	19	0	15.8	36.84	36.84
Sample 10	6/7/2016	3	4	9	10	21	14.29	19.04	42.86	47.62
Sample 11	6/14/2016	2	3	3	5	21	9.5	14.29	14.29	23.81
Sample 12	6/14/2016	2	4	6	8	19	10.5	21.05	31.58	42.11
Sample 13	6/14/2016	0	2	2	4	21	0	9.52	9.52	19.05
Sample 14	6/14/2016	0	0	2	2	24	0	0	8.33	8.33
Sample 15	6/14/2016	0	0	2	2	23	0	0	8.7	8.7
Sample 16	6/14/2016	0	1	3	5	22	0	4.55	13.64	22.73
Sample 17	6/14/2016	0	0	3	7	22	0	0	13.64	31.82
Sample 18	6/14/2016	1	1	6	10	24	4.17	4.17	25	41.67
Sample 19	6/14/2016	0	1	2	4	23	0	4.35	8.7	17.39
Sample 20	6/14/2016	0	2	2	4	23	0	8.7	8.7	17.39

260-2 *ΔfgsiR34* +	Inoculation date	dpfi				Number of Spikelets	FDK (%)			
		7	14	21	28		7	14	21	28
Sample 1	6/7/2016	0	0	0	2	23	0	0	0	8.7
Sample 2	6/7/2016	0	0	0	0	24	0	0	0	0
Sample 3	6/7/2016	0	0	2	2	26	0	0	7.69	7.69
Sample 4	6/7/2016	0	0	0	4	25	0	0	0	16
Sample 5	6/7/2016	0	2	2	3	25	0	8	8	12
Sample 6	6/7/2016	0	0	0	2	28	0	0	0	7.14
Sample 7	6/7/2016	0	0	0	3	23	0	0	0	13.04
Sample 8	6/7/2016	0	0	2	4	24	0	0	8.33	16.67
Sample 9	6/7/2016	0	0	2	4	22	0	0	9.09	18.18
Sample 10	6/7/2016	0	0	2	4	25	0	0	8	16
Sample 11	6/14/2016	0	2	2	5	24	0	8.33	8.33	20.83
Sample 12	6/14/2016	2	2	4	6	25	8	8	16	24
Sample 13	6/14/2016	0	2	3	5	22	0	9.09	13.64	22.73
Sample 14	6/14/2016	0	4	8	16	25	0	16	32	64
Sample 15	6/14/2016	0	2	4	8	20	0	10	20	40
Sample 16	6/14/2016	0	2	7	7	23	0	8.7	30.44	30.44
Sample 17	6/14/2016	0	1	6	12	25	0	4	24	48
Sample 18	6/14/2016	2	5	6	6	23	8.7	21.74	26.09	26.09
Sample 19	6/14/2016	0	1	3	3	22	0	4.35	13.64	13.64
Sample 20	6/14/2016	0	0	4	12	22	0	0	33.33	54.54

260-4 wildtype	Inoculation date	dpfi				Number of Spikelets	FDK (%)			
		7	14	21	28		7	14	21	28
Sample 1	6/7/2016	2	6	10	15	23	8.7	26.1	43.48	65.22
Sample 2	6/7/2016	0	4	9	14	24	0	16.67	37.5	58.33
Sample 3	6/7/2016	1	3	15	17	27	3.7	11.11	55.56	62.96
Sample 4	6/7/2016	0	4	16	18	26	0	15.39	61.54	69.23
Sample 5	6/7/2016	0	3	12	16	26	0	11.54	46.15	61.54
Sample 6	6/7/2016	0	4	15	18	28	0	14.29	53.57	64.29
Sample 7	6/7/2016	0	0	5	7	27	0	0	18.51	25.92
Sample 8	6/7/2016	0	0	4	8	28	0	0	14.28	28.57
Sample 9	6/7/2016	2	4	6	10	24	8.33	16.66	25	41.66
Sample 10	6/7/2016	2	4	4	9	24	8.33	16.66	16.66	37.5
Sample 11	6/14/2016	3	6	12	16	25	12	24	48	64
Sample 12	6/14/2016	3	7	18	22	22	13.63	31.81	81.81	100
Sample 13	6/14/2016	4	8	16	20	22	18.18	36.36	72.72	90.90
Sample 14	6/14/2016	1	3	4	8	19	5.26	15.78	21.05	42.10
Sample 15	6/14/2016	0	1	3	7	21	0	4.761	14.28	33.33
Sample 16	6/14/2016	2	4	7	12	24	8.33	16.66	29.16	50
Sample 17	6/14/2016	8	11	15	18	20	40	55	75	90
Sample 18	6/14/2016	0	2	4	6	23	0	8.69	17.39	26.08
Sample 19	6/14/2016	0	1	4	8	23	0	4.34	17.39	34.78
Sample 20	6/14/2016	0	2	8	10	25	0	8	32	40

| 260-4 *ΔfgsiR34+*	Inoculation date	dpfi				Number of Spikelets	FDK (%)			
		7	14	21	28		7	14	21	28
Sample 1	6/7/2016	0	0	2	3	22	0	0	9.09	13.64
Sample 2	6/7/2016	0	3	4	5	22	0	13.64	18.18	22.73
Sample 3	6/7/2016	0	0	0	3	23	0	0	0	13.04
Sample 4	6/7/2016	2	4	5	8	20	10	20	25	40
Sample 5	6/7/2016	0	4	4	8	24	0	16.67	16.67	33.33
Sample 6	6/7/2016	0	2	4	6	26	0	7.69	15.38	23.08
Sample 7	6/7/2016	0	0	2	5	23	0	0	8.7	21.74
Sample 8	6/7/2016	0	1	1	2	24	0	4.17	4.17	8.33
Sample 9	6/7/2016	0	0	0	4	26	0	0	0	15.38
Sample 10	6/7/2016	1	5	5	12	22	4.54	22.73	22.73	54.55
Sample 11	6/14/2016	0	2	5	6	26	0	7.69	19.23	23.08
Sample 12	6/14/2016	0	2	6	12	25	0	8	24	48
Sample 13	6/14/2016	0	4	5	12	25	0	16	20	48
Sample 14	6/14/2016	2	4	4	7	22	9.09	18.18	18.18	31.82
Sample 15	6/14/2016	0	1	2	5	25	0	4	8	20
Sample 16	6/14/2016	0	0	3	7	24	0	0	12.5	29.17
Sample 17	6/14/2016	2	3	5	12	25	8	12	20	48
Sample 18	6/14/2016	4	4	7	8	22	18.18	18.18	31.82	36.36
Sample 19	6/14/2016	2	2	4	6	22	9.09	9.09	18.18	27.27
Sample 20	6/14/2016	4	6	6	8	24	16.67	25	25	33.33

Appendix 5. Copy number of *Tri6* genes of interest and total mass of DNA required for each PCR reaction to set up a standard curve

Copy number of tri genes of interest	Mass of haploid genome	Total DNA mass (pg) to be used in PCR reaction to set up a standard curve
10,000,000	0.039	390000 (390 ng)
1,000,000	0.039	39000 (39 ng)
100,000	0.039	3900 (3.9 ng)
10,000	0.039	390 (0.39 ng)
1000	0.039	39 (0.039 ng)

Appendix 6. NCBI blast hit showing the position of *fgsiR34* upstream of *Tri5* gene in the core cluster in chromosome 2 of *F. graminearum*

fgsiR34 **seed region**
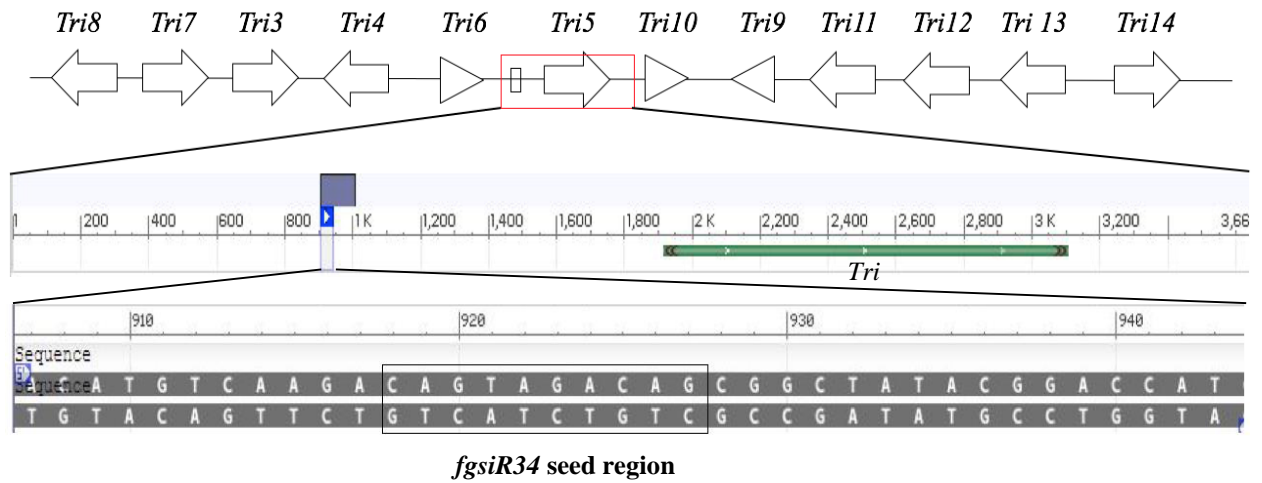
Appendix 7. Sequence of *fgsiR34* potential target site on chromosome 2 of *F. graminearum*

Seed region (2-11 bases) of *fgsiR34* maps 997 bases upstream of *Tri5* gene
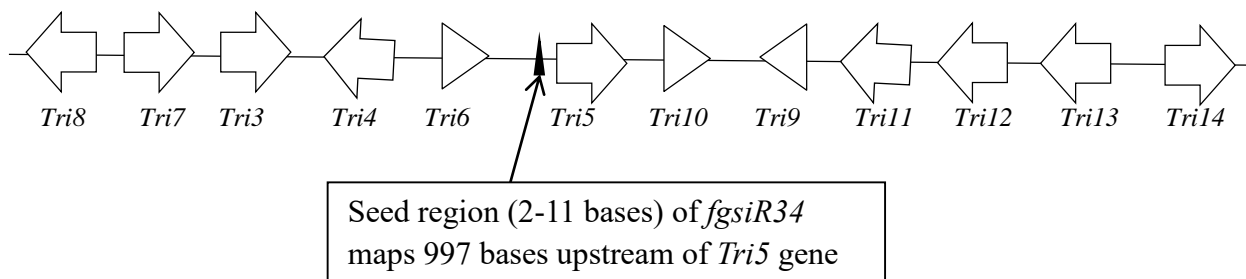
>gi|22087476|gb|AF508152.1| *Gibberella zeae* isolate A18 trichodiene synthetase (Tri5) gene, complete cds

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AAGCTTTGCGCGGTCAGTCAAATTCGCACATGTCGATACTTTTTGCTGGCATTGGCTCAGTATTCATCTA
TGAGTGGCTGAACCGTAGTTGATTCTCAAGTCCAACCCTAATAGTGCCCGGCGGAATGAGACGTTTTCGG
GTGTCTGTAGCCGAGATGCGGATAGTAACGGTAACCCTAGTCAAATGAGACATGGGCAGGGTTCATGGTT
GTTGAACCTTGTTCATCAGAATGTTGATGCCGTTGACCTACGGAATACCATCTTTCACATGTATTTGTTC
CCAACCCACGTGGCTATACCAACATCCGCTGCATACTGATTGGTTGGCTTCATATATTGATACAGGTAAG
TTCAAGATCGCGAGCGGGAATTTCCTTATCAAATCAGTGAGATGCTCTACGCCGTGGGGGTAAAAGTCAA
ATCAGATAAGAGCACAGCAACCGGCAATGCTCACGGGCTACAGTGAATGTTCGTGATATGATTGTGGCTT
CCTCTATATCACTCACATATCCACAATTATTCCAAACAGTTCGAGTTCTGTAGATAGTAGATACGTCTTC
CAGGATGTATATAATCTAGCAGCCGGTAGTTGAAACACCTACCATGTAGAGGCGAGGAGCGCAGCATCGC
CAGTATGCACGAAGTACGGATATGATGATCTCAATTGTGGTTCCCCTGCTGCTAGTGCGACCGGACTTGT
CACAGTTGCTGTATTCCCAAATTATGTCAGTTTCTGTCAGCGATGACAGGCATGCCAGATAACCGTTCTC
GTCATCATCGCCGTTAGATGGACAAGGGCAGCGTAGCTTTAAACTGTGAAAACGGTTGCATGCATGCTGC
ACGTGTAGATTCCTATGGCGTGAACACCCCTACCATGAACACCCTGATATCTTCGCATAGATAAGGACAT
GTCAAGACAGTAGACAGCGGCTATACGGACCATGTCCTAGAACTAAGACATGGGTTAATTGAAAACGGGA
TTCTGTACTCTGTACAGAATACAGAGTATTTCTTCGACATGGGGGAATAACCACCGTTTCCCGCCCCTTG
TAAAGCTAAGCGTTTTTAATATGGAAAACGGGGTTCATCTACAGTAGAGTCGACAAGATCTGCAATCTAT
CAATGCTTAAATGCCGTTCCAGCTACGCGACTTTCAGGCACCGGCAATGGAGATCTTGGCTAAGATTCTA
GTCTAACTAACACACAGGAAATGGGTTTTAGATCCTGCTAACCCTAAAAGGGCTAAGTTGCCCGAACCTT
ATTCAACGCAGTTGCAGTGCATTCGGGAGCCAACTCCGCGGGATATATGTGATGGCCGGCAGTTTTGTAG
TGCTGATCATAAAAGTGGTCATATTTAAGGCCTGTCGCCCAGGTAGACTTTTGCCAGGGCACAATATAAC
ACCGCGACTATCTAGGTAAGTGAGGCTTTCTTCTGTATTGATAGTCGGTCGTTTCACTAGTCCAAACACA
GACCACGGACAATGACCGAACTCAATATCCCGGTCCAAGGATTGATCCCTAGATTTAGGCCTACTCCCGG
CCCTTTGATACTAGCATCTAGCACCAATCGCTTTTGTAGGTCTTCCAAGTGTGTCGAGCTAAAGACAAAA
TGAACCAAGAGTTTGTTCCAAGAGCCGGATATTTTCTGACATCTATAGCCTTGCAGGGAATGAGAGAGCA
CGTCCATACGTCAAAGTCTCTCTTTACGACCGTCCGGTTGGGGACGCTATTTGCATTGACTTTGGATCAG
TCTTTTAGGCCTAACAATACAATATCGACTAATAGATGTGTACGGGTCGAGATGTTTGCCATCTATTGATA
TATAGTTAATAGC                                           CTTGTTGATTTATTGAATCA
TTGTTACCAGTAC
ATGGAGAACTTTC
CTTCTCGAGTATA                                           CATTGAGAATTTGCACTATG
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Potential binding site of *Tri6* transcription factor (it binds to TNAGGCCT consensus sequence in the promoter of *Tri* cluster)

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CTTACAACAAGGCCGCCCATCACTTTGCTCAGCCTCGCCAACAGCAGATGCTCAAAGTAGACCCTAAGCG
ACTACAGGCTTCCCTCCAAACAATCGTTGGTATGGTCGTATACAGCTGGGCAAAGGTCTCCAAAGAGTGC
ATGGCGGATCTATCTATTCACTACACCTATACTCTCGTTTTGGATGATAGCAGCGATGACCCGCATCCTG
CCATGTTGAACTATTTTGACGACCTTCAAGCCGGACGAGAGCAGGCCCATCCATGGTGGGCACTTGTCAA
CGAACACTTTCCCAACGTCCTTCGCCATTTTGGACCCTTCTGCTCATTGAACCTTATCCGTAGCACTATG
GACTGTGAGTAGTAACTCAATTATATTATTTTCCTATATGAAGAGGCTGACGACAGTGGAACAACAGTTT
TTGAGGGATGCTGGATTGAGCAGTACAACTTTGGAGGATTCCCGGGCTCTGATGACTACCCTCAATTCCT
TCGACGCATGAACGGCTYGSGTCATTGTGTTGGGGCTTCTCTATGGCCCAAGGAGCTGTTCGACGAGCGA
AAGAATTTCCTCGAAATCACGACAGCCGTTGCCCAGATGGAGAACTGGATGGTTTGGGTCAATGATCTCA
TGTCATTCTACAAGGAATTCGACGATGAGCGTGACCAGATCAGTCTGGTCAAGAACTTTGTCACCTGTCA
TGAGATCACTCTGGATGAAGCTTTGGAGAAGCTCACGCAGGAAACTCTGCACTCATCCAAGCAGATGGTT
GCTGTCTTCGCGGATAAGGACCCTCAGGTGATGGACACGATTGAGTGTTTCATGCACGGCTACGTTACGT
GGCATTTGTGCGACGCCAGATACCGCCTCCATGAGATTTATAAAAAGGTCAAGGATCAAGATACAGAGGA
CGCTAAGAAGTTCTGCAAGTTCTTTGAGCAGGCAGCCAATGTCGGCGCTGTTGCACCCTCGGAGTGGGCT
TATCCACAAGTTGCACAACTGGCAAACGTTCGGGCCAAGGGCGATGTGAAGGAGGCTCAGAAGCCCATCC
TAAGTTCAATTGAGCTAGTGGAGTAACCGAAGGCGAGTTTGGAAGTATGTTTTGCGGGTACGGGTAGTCG
TTTGGAGAATGGTGGTCTGTTATAATGATTACAAATAGTTTAGTTGTGTTTTCATTACATAGAATGAACA
GTTGAACAAGGATAATTACTTCGGAATAGGCAGTTGAAACTGAATGGATGTATGTAACCTGAGCCTGTAA
CCATTTCCCACTCGAGTGCAGGCTTTTGCGTAACCAAGTCTGTACACCCGTCGGTGCGACAGGGCTACCC
CCAACCCTGCAACTGCAGCTGCAGCTGGCAGACTGGCGCTACGAATTGTAGTGCGATGCGGGCATCTTGT
ACCCGCTCGGAGGTTGGAGGGAGCTGGCACATTCTTTAGACCCGCGAATTGATCTTCAAAGCGCTTGCGT
TTTGTCCAGATCAGTGACCATACCCTTGCCTTCCCGCACCACCCAAACGTCAAGTGAACGAGGCGTACAG
AAACCACACAAGATAAGGTTTAATGCCTGCTTGAGCACTATGAGGGACACGACACTGCTGTAAAACTCTA
TCCTTGCATTATATTGTAACATCGTTTAA
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Tri5 gene

      Primers for MSP were designed on the highlighted sequence that spanned the seed region. A 264 bp amplicon obtained using unmethylated primer pair was cloned into pGEM-T vector and sent for sequencing to GenScript. The sequence result showed conversion of all the cytosine residues to thymines, including the cytosines on the CpG region. This showed that the seed region of *fgsiR34* at 997 bp upstream of *Tri5* gene was unmethylated in both the wildtype and the *ΔfgsiR34+* strains of *Fusarium graminearum*.

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TGTTAGTGATGATAGGTATGTTAGATAATTGTTTTTGTATATTGTTGTTAGATGGATAAGGGTAG
TGTAGTTTTAAATTGTGAAAATGGTTTTATGTATAATGTATGTGTAGATTTTAATGGTGTGAATA
TTTTTATTGTGAATATTTTGATATTTTTGTATAGATAAGGATATGTTAAGATGGTAGATAGTGGT
TATATGGATTATGTTTTAGAATTAACATATGGGTTAATTGAAAATGGCATTTT
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