

Identification of molecular determinants involved in compatible and incompatible interaction of wheat-*Fusarium graminearum* using integrated-omics approach

A thesis submitted during the year 2025 to the University of Hyderabad
in partial fulfillment of the award of a Ph.D. degree in the Department of Plant Sciences, School
of Life Sciences

by

(Massarat Fatima)

(Registration No: 17LPPH09)



Department of Plant Sciences, School of Life Sciences

University of Hyderabad

Gachibowli, Hyderabad –500 046

Telangana

India



CERTIFICATE

This is to certify that the thesis entitled **“Identification of molecular determinants involved in compatible and incompatible interaction of wheat-*Fusarium graminearum* using integrated-omics approach”** submitted by Massarat Fatima bearing Registration Number: 17lpph09 in partial fulfillment of the requirements for the award of Doctor of Philosophy in the School of Life Sciences, is a bonafide work carried out by him/her under my supervision and guidance.

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1. **Genome-wide search and gene expression studies reveal candidate effectors with a role in pathogenicity and virulence in *Fusarium graminearum*.**

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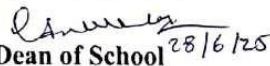
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3. PL 803	Research Proposal and Scientific Writing	4	Pass
Total Credits		12	Pass


Supervisor
27/06/2025

RAGIBA MAKANDAR Ph.D.
Professor
Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500 046, India


Head of the Department
27/06/2025


Dean of School
28/6/25

संकाय अध्यक्ष / DEAN
जीव विज्ञान संकाय / School of Life Sciences
हैदराबाद विश्वविद्यालय / University of Hyderabad
हैदराबाद / Hyderabad-500046,

DECLARATION

I, Massarat Fatima hereby declare that this thesis entitled “**Identification of molecular determinants involved in compatible and incompatible interaction of wheat-*Fusarium graminearum* using integrated-omics Approach**” Submitted by me under the guidance and supervision of Professor/Dr. Ragiba Makandar is a bonafide research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. I hereby agree that my thesis can be deposited in Shodhganga/INFLIBNET. A report on plagiarism statistics from the University Librarian is enclosed.

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Student Regd. No.: 17LPPH09

Supervisor(s): Prof. Ragiba Makandar

Department/Centre: Department of Plant Sciences

School of Life Sciences

Among 17 goals (<https://sdgs.un.org/goals>), under which SDG the work incorporated in the thesis will be addressed: **(SDG2)-Zero Hunger (Agriculture and sustainable development)**

Abstract

This study aligns with Sustainable Development Goal (SDG) 2: Zero Hunger, which emphasizes eradicating hunger, achieving food security, improving nutrition, and promoting sustainable agriculture. Our research on plant-microbe interactions and disease management directly supports initiatives aimed at enhancing agricultural productivity and resilience against fungal pathogens, thereby contributing to global food security. This study plays a crucial role in Sustainable Development Goal (SDG) 2 by addressing major agricultural challenges, such as fungal infestations, that threaten crop yield and quality. Investigating plant-microbe interactions and disease management represents a significant contribution to sustainable agriculture. Global food security is increasingly jeopardized by fungal pathogens that adversely impact crop yield and quality. In addressing this challenge, plant-microbe interactions offer innovative disease management strategies that enhance resistance and sustainability in agriculture. This research explores biological solutions to mitigate fungal infestations, promoting eco-friendly practices that reduce reliance on chemical fungicides. This approach diminishes dependence on chemical fungicides, fostering environmentally sustainable and resilient agricultural practices. Healthier crops lead to higher yields and better food quality, thereby improving and directly supporting efforts to feed growing populations. By enhancing crop health and productivity, these strategies align with the Zero Hunger Challenge, ensuring more resilient food systems.

Dedicated to my parents

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Plagiarism Report

ABBREVIATIONS

%	Percent
°C	Degree Celsius
μL	Microlitre
μM	Micromolar
pmol	Picomoles
μg	Microgram
ng	Nanogram
mL	Mililitres
mM	Milimolar
nm	Nanometer
g	Grams
mg	Miligrams
v/v	Volume by volume
w/v	Weight by volume
Psi	Pounder per square inch
hr	Hours
min	Minutes
s	Seconds
T _m	Annealing Temperature
hpi	Hour's post-inoculation
hpt	Hour's post-treatment
dpi	Days post-inoculation
dpt	Days post-treatment
rpm	Revolutions per minute
bp	Base pairs
siRNA	Small interfering Ribonucleic acid
dsRNA	Double-stranded RNA
CTAB	Centrimethylammoniumbromide

EDTA	Ethylene diamine tetraacetic acid
PDA	Potato Dextrose agar
CMC	Carboxymethylcellulose
DEPC	Diethylpyrocarbonate
ITS	Internal transcribed region
NT	Nobeoka treated
cDNA	Complementary DNA
PCR	Polymerase chain reaction
qPCR	Quantitative Polymerase chain reaction
RT-qPCR	Real-time Quantitative Polymerase chain reaction
ANOVA.	Analysis of variance
SE	Standard error
DEG	Differentially expressed genes
ROS	Reactive oxygen species
NC	Nobeoka control
DON	Deoxynivalenol
CRP	cAMP regulatory protein
FHB	Fusarium head blight
CFEM	Common in fungal extra-cellular membrane
Kp	Killer protein
OMA	Oat Meal Agar
URC	Unrelated control
Fg	<i>Fusarium graminearum</i>
Mg	<i>Magnaporthe oryzae</i>
ACT	Actin
TRI	Trichothecene
SC	Sonalika control
ST	Sonalika treated
TEF	Translation elongation factor

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- Table S1** Details of primers used to amplify and sequencing of the internal transcribed spacer (ITS), D1-D2 region of the 28S region of nuc rDNA (D1-D2), and *TEF1* gene, along annealing temperature, expected amplicon size, and accession number.
- Table S2** The partial nucleotide sequences of candidate effector and non-effector genes of *F. graminearum* strain TN01.
- Table S3** The consensus motif sequences detected in the effector genes of *F. graminearum* characteristic of their gene structure.
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Chapter. 1

Research Purpose and Objectives of Study

1 INTRODUCTION

Fusarium head blight (FHB), also known as head scab or scab, is predominantly caused by *Fusarium graminearum* (sexual state *Gibberella zeae*) and represents a significant threat to cereals such as wheat and barley. The FHB disease, thrives in moist and humid climates, and its impact may be destructive within weeks of harvest. The primary consequences of FHB include reduced yields, shriveled grains, mycotoxin contamination, and diminished seed quality (McMullen et al., 1997). The fungus directly reduces grain yield through blight, while indirectly causing losses via the accumulation of mycotoxins, particularly deoxynivalenol (DON), which pose health risks to both humans and livestock (Bai et al., 2001). The inoculum sources for FHB development are primarily crop debris and alternative hosts such as grasses and weeds. Given the widespread nature of FHB and the complexity of its development in hosts, both the disease and its principal causative agent have been subjects of extensive research. The genome of *F. graminearum* has been sequenced and annotated (Cuomo et al., 2007), and is available in detail at Ensembl Fungi, offering valuable insights for studying gene functions and conducting comparative analyses with other species.

1.1 History of Fusarium head blight at Global and in the Indian Context

Fusarium head blight (FHB) was initially documented in England by Smith (1884). Major wheat producers in FHB-prone agricultural regions around the world are particularly vulnerable to FHB. These places experience significant losses as a result of frequent and severe FHB outbreaks. For many years, cereal crops in the United States have been most threatened by FHB. Between 1993 and 2014, the United States suffered a substantial loss of USD 17 billion due to FHB on wheat. Since 1950, China has experienced 30 FHB epidemics, each resulting in more than 10% loss of total land (Moonjely et al., 2023). Although *F. graminearum* is the most frequently encountered causative agent of FHB in North America (Gilbert et al., 2000), several other *Fusarium* species

have also been isolated from the infected small grains worldwide. While *F. graminearum* is predominant in hotter and humid regions of the world, including parts of North America, Canada, Australia, and Central Europe. Several species of *Fusarium* have been reported to cause the disease. Despite efforts to mitigate FHB incidences over the past decade, FHB infection and DON accumulation in grains have continued to cause significant economic losses (McMullen et al., 2012). Since 2016, FHB has gradually moved northward and is now a prevalent disease infecting wheat in China. The big outbreak in 2012 caused the devastation of around 10 million hectares of wheat cultivation and a loss of over 2 million tons in yield (Powell et al., 2021). The disease occurs persistently worldwide, including Brazil, Canada, Russia, Eastern and Western Europe, and Romania, accounting for over 50% of global production (Ghimeri et al., 2022).

Though FHB is not currently significant in India. However, due to substantial changes in agronomic conditions with rising temperatures, increased rainfall, and humidity during flowering, FHB is making wheat crops vulnerable and is becoming increasingly predominant in India as well. In northern India, warm and humid conditions during March-April, can lead to FHB occurrences, particularly affecting wheat and barley crops in Punjab, Haryana, Bihar, and Himachal Pradesh. Consequently, FHB could soon pose a major threat to Indian agriculture in the main wheat-producing regions. The disease was first identified in India in the Siang District of Arunachal Pradesh (Roy et al., 1973). Later, researchers reported it in Wellington and Gurdaspur, Punjab. Bagga. (2008) noted that the disease severely affected wheat cultivar PDW 274 in Dera Baba Nanak, Gurdaspur, causing significant yield losses. Recent reports caution that FHB will likely increase with reduced tillage-based wheat cultivation, worsened by climate change, particularly in northern and southern regions such as the Nilgiris (Saharan et al., 2021).

1.2 Genome biology of *F. graminearum*

The classification of *F. graminearum* is described in the (Table 1.1). The genome of *F. graminearum* was sequenced and annotated, giving a total genomic size of 36.1 Mb. (Cuomo et al., 2007). This relatively small genome size may be attributed to the limited presence of highly identical duplicated sequences due to genome expansion. The *F. graminearum* genome as per Genome Database (<http://mips.gsf.de/genre/proj/fusarium>), encodes 13,826 genes. From the total genes, 1,720 genes exhibit high similarity to known genes, 4,058 show similarity to known genes, 4,915 demonstrate some similarity to unknown genes, and 3,133 display no similarity to any genes in other organisms (Wong et al., 2011). Unlike the closely related species *Fusarium oxysporum* and *Fusarium verticillioides*, *F. graminearum* has undergone extensive chromosomal rearrangements. Notably, *F. graminearum* has just four chromosomes, but *F. verticillioides* has eleven, and *F. oxysporum* has eleven core chromosomes and four lineage-specific (LS) chromosomes (Ma et al., 2010).

Table 1.1 Classification of the fungal pathogen *F. graminearum*

Scientific Classification	
Kingdom	Fungi
Subkingdom	Dikarya
Division	Ascomycota
Subdivision	Pezizomycotina
Class	Sodariomycetes
Sub Class	Hypocreomycetidae
Order	Hypocerales
Family	Nectriaceae
Genus	<i>Fusarium</i>
Species	<i>Graminearum</i>

1.3 Life cycle and pathogenesis of *F. graminearum*

Warm and humid conditions are most favorable for FHB infection. During anthesis and throughout kernel development, wheat spikes are particularly prone to *Fusarium* infection. The fungus's asexual conidia or sexually developed ascospores are widely disseminated by wind or rain, reaching the exposed anthers of the flower and so initiating the infection cycle. Although ascospores, macroconidia, chlamydospores, and hyphal fragments can all serve as inoculum, the primary inoculum that causes epidemics is the ascospores discharged from crop detritus (Bai & 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 as described in (**Figure 1.1**) from where they germinate and initiate infection (Trail, 2009).

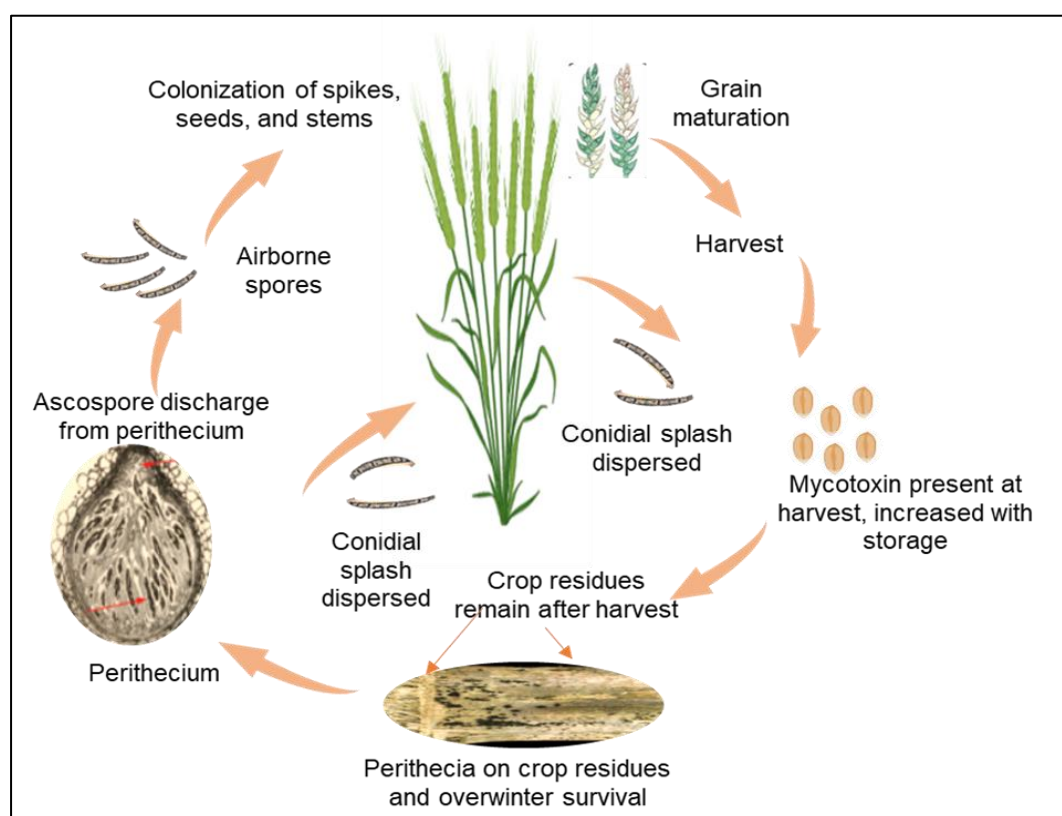


Figure 1.1 Disease cycle of Fusarium head blight of wheat caused by *F. graminearum*.

The fungus overwinters on crop debris, where perithecia grow. Perithecia then produce spores that settle on neighboring plants. The fungus initiates infection in the extruded anthers and subsequently spreads throughout the caryopsis, floral bracts, and rachis (Bai & Shaner 1994; Bushnell et al., 2003). It can also begin infecting by directly penetrating the glumes, palea, or rachilla of the wheat floret. Infected florets develop dark-brown, water-soaked spots on the glumes, leading to blight. The infection then extends to other spikelets via the vascular bundles of the rachilla and rachis. Ultimately, the florets either fail to produce grain or yield poorly filled grain (Bushnell et al., 2003).

1.4 Fusarium head blight symptoms development

The Initial visible lesions typically appear on the first florets between 2 to 4 days after infection, often around the center of the head (Andersen, 1948). Under conditions of suitable temperature and moisture, these lesions exhibit a water-soaked appearance and range in color from purplish to brown, with a bleached center. As time progresses, they become more saturated with water, darken to an olive-green hue, and extend to the rachis (Atanasoff, 1920). As the infection persists, the fungus produces macroconidia, which give the surfaces of florets and glumes a pinkish tint. Eventually, the lesions grow larger, resulting in the blighting of the entire florets (**Figure 1.2**).



Figure 1.2 Typical image showing (A) growth of *F. graminearum* on potato dextrose agar (PDA). (B) FHB-infected wheat spikes. (C) FHB- infested wheat grains.

As the disease advances, the fungus can extend along the length of the rachis and the spikelets within the spike (Bai & Shaner, 2004). The grains from infected FHB spikes appear “tombstone” producing low test weight and chaffy seeds causing severe effects if the infection is early (Bushnell et al., 2003).

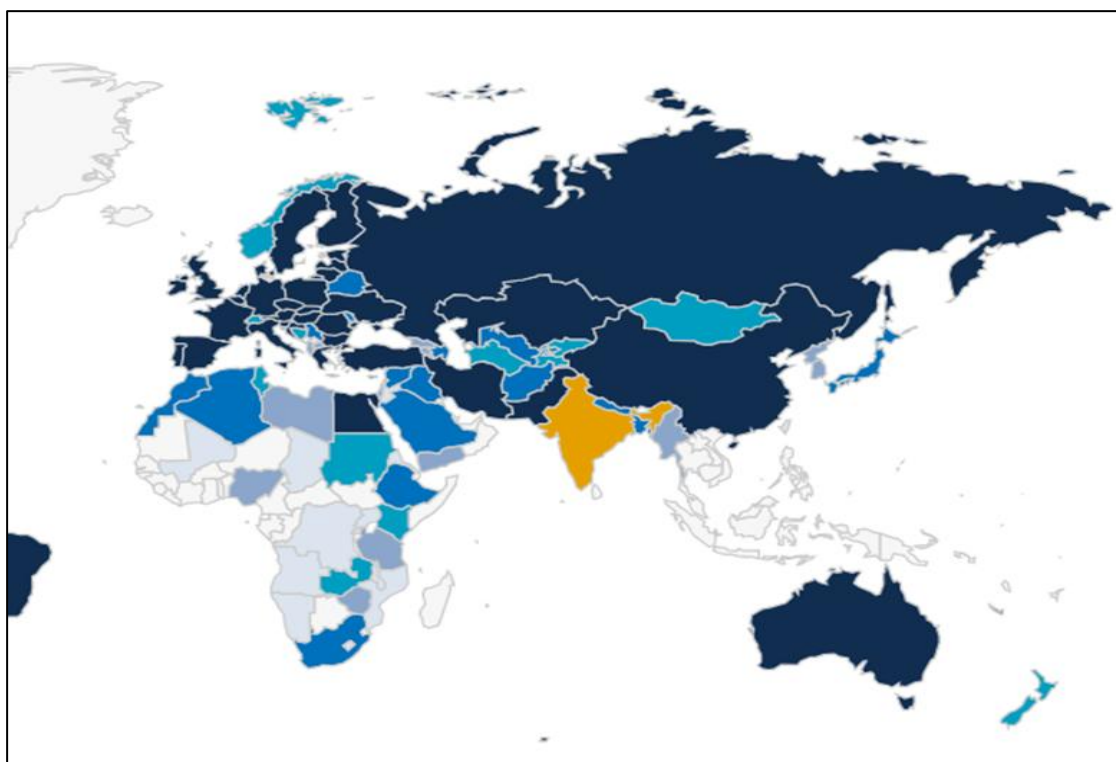
1.5 Importance of wheat crop and its disease

Wheat (*Triticum aestivum*, L), a pivotal cereal crop is extensively cultivated globally due to its significance as a primary food source and its exceptional suitability for bread production. Enhancing its yield and quality remains a primary focus in agricultural research. The genus wheat belongs to the grass family, Poaceae that comprises various cultivated species, hexaploid *T. aestivum* being the major one ($2n = 42$) with the genome set represented as AABBDD. The classification of wheat is described in (Figure 1. 3).

Scientific Classification	
Kingdom	Plantae
Clade	Angiosperms
Clade	Monocots
Clade	Commelinids
Order	Poales
Family	Poaceae
Subfamily	Pooideae
Genus	<i>Triticum</i>
Species	<i>Aestivum</i>

Figure 1.3 Pictorial representation and scientific classification of wheat (*Triticum aestivum*, L.)

The total global wheat production for the year 2023-2024 has been estimated at 798 million tons (<https://www.fas.usda.gov/data/production>). The average global wheat productivity is 4.36 metric tons/hectare, as described in **(Figure 1.4)**. Throughout its growth and development, wheat yield is influenced by both biotic (pests and pathogens) and abiotic stresses. Biotic stress is a well-researched domain, of which fungal pathogens are a major concern for crop growth and development, thereby significantly impacting the yield of the world's most essential cereal crops, particularly wheat. China, European Union, and India being the three major global producers of wheat. India ranks third in wheat production after European Union (EU) and China.



S. No	Market	% of global wheat production	Total production (2023/2024) Metric tons
1	China	17 %	136.59 million
2	European Union	17 %	134.87 million
3	India	14 %	110.55 million

Figure 1.4 Map showing the global percentage of area and production of wheat crop. (<https://fas.usda.gov/data/production/>).

The resistance to *Fusarium* is categorised into two kinds in wheat (Schroeder & Christensen, 1963): Type I resistance-initial infection resistance, whereas Type II resistance, which is resistance spreading of infection within a wheat head. It has been observed previously (Steffenson et al., 2003) that a Type II resistance to *Fusarium* by wheat cultivars (Bushnell, 2002), whereas Type I resistance exists in other cereals such as barley. In wheat, multiple inoculation techniques have been utilised to contrast these two kinds of resistance. To identify Type I resistance, spore suspension is sprayed on wheat heads, and blighted spikelets are counted following inoculation. Point inoculation of the spikelets is carried out to measure Type II resistance. The other types of resistance by physiological alterations are also considered which include resistance to DON accumulation, kernel and *Fusarium* head blight infections (Mesterhazy, 1995). Sumai 3, a Chinese cultivar, exhibits type II resistance and is considered the elite source of resistance in wheat breeding programs (Bai & Shaner, 2004).

However, FHB resistance in wheat is a complicated, quantitative trait that is frequently associated with negative agronomic characteristics such as tiny heads, tall stature, and late maturity. Given the significant toxicity of *Fusarium* mycotoxins and the negative impact of FHB on wheat yield, it is vital to adopt plant protection approaches such as crop rotation, the adoption of resistant cultivars, and crop practices. Under certain environmental conditions, the use of fungicides becomes critical to maintaining wheat productivity and producing high-quality grains. For a better understanding of the epidemiological factors and to execute plant protection measures, it is critical to explore and develop novel approaches for early prediction and detection of FHB outbreaks in cereals.

1.6 Importance of mycotoxins in *F. graminearum*

The generation of mycotoxins by *Fusarium graminearum* is a serious problem since it can contaminate wheat grains. In addition to reduced grain yield, the fungus creates mycotoxins in

infected grains. Mycotoxins, secondary metabolites produced by the fungus in infected grains, pose a health risk to humans and animals (Mankevičienė et al., 2007). The presence of mycotoxins in the host is influenced by several factors, including the nature of fungal strain, climatic and geographical conditions, host plant susceptibility, cultivation techniques, and crop protection practices. during storage (Pancaldi et al., 2010). The mycotoxin DON is reported to contribute as a significant virulence factor produced by *F. graminearum* during the wheat head interaction (Desjardins et al., 1993).

F. graminearum infects wheat spikelets and spreads throughout the head, causing a significant increase in DON production. The Trichothecene genes responsible for its control have been uncovered in *F. graminearum* (Brown et al., 2001; 2002). DON inhibits protein synthesis by attaching to the eukaryotic ribosome's 60S subunit and intervening with peptidyl transferase (Ehrlich & Daigle, 1987). Apart from DON, *F. graminearum* produces several mycotoxins, including 15-acetyl deoxynivalenol (15-ADON), 3-acetyl deoxynivalenol (3-ADON), nivalenol, and zearalenone. Chemically, DON a one of the polar compounds of the trichothecene family. Aside from their hazards to animals and phytotoxicity to cereal crops, trichothecenes also operate as virulence factors in the head blight of maize and wheat produced by *F. graminearum* (Jansen et al., 2005). Recent researches have revealed other genes and signaling pathways that regulate DON production, in addition to *TRI* genes.

1.7 Detection of effectors *F. graminearum*

Pathogenic fungi use a variety of small secreted proteins or molecules to manipulate host cell structure, metabolism, defensive responses, and other cellular processes to infect them. *F. graminearum* is noted for its production of a diverse array of effectors, which are essential for the successful invasion and colonization of host plants. These effectors are cysteine-rich small proteins with signal peptide sequences and the absence of the transmembrane domain, secreted by

the fungus during infection in the host plants (Lysoe et al., 2011). Some effectors, designated as cytoplasmic effectors, are transported into the host cells where they directly influence cellular functions, while others, referred as apoplastic effectors, operate in the extracellular space within plant tissues. Effectors not only compromise plant defenses but also create conditions conducive to pathogen colonization and growth (Lovelace & Ma, 2022). Collectively, effectors are vital for disease development and contribute to susceptibility of the plants to the fungal pathogens.

Due to the co-evolution of hosts and pathogens, plants have evolved immune receptors capable of recognizing specific effectors and triggering immune responses (Jones & Dangl, 2006). These effectors can modify physiology of the plants to benefit the fungus by facilitating penetration and colonization of host tissues thereby suppressing defense mechanisms, and altering plant metabolism. Gene editing can impair several effectors that promote *F. graminearum* proliferation, potentially increasing wheat resistance to FHB. In contrast, some effectors dampen the plant defense responses by targeting resistance genes. These effectors act as critical components of plant-pathogen interactions, and their presence and activity can determine the outcome of an infection.

The major function of effectors is to dampen immune responses produced by pathogen-associated molecular patterns (PAMPs) (Lacomme & Santa Cruz, 1999). In the battle to acquire arms to take over pathogens, plants have acquired pattern recognition receptors (PRRs) that recognize PAMPs and activate PAMP-triggered immunity (PTI). The effectors act as PTI-disrupting components, dampening PTI in the host while enhancing the spread of pathogens in the host, a phenomenon known as effector-triggered susceptibility. On the other hand, when the effector molecules are detected by the corresponding receptors/resistance components, which comprise R proteins or nucleotide-binding leucine-rich repeat receptors (NLRs), effector-triggered immunity (ETI) (Chen et al., 2018), resulting in an incompatible interaction with resistance as the outcome. Whereas, the

absence of matching resistance (R) and avirulence (Avr) components may result in a compatible interaction with susceptibility as the outcome. The research findings revealed the expression of the effector and non-effector gene candidates, which could serve as both pathogenicity and virulence genes, indicating a complex mechanism employed by the pathogen to suppress PTI and ETI and promote its infection in the host.

Effectors from *F. graminearum* have been studied using various techniques, including genome sequencing, gene expression profiling, proteomics, and biochemical assays. These investigations have demonstrated that these effectors are highly diverse, with some involved in modulating plant defense responses, while others are engaged in host cell invasion and growth. Additionally, some effectors participate in the breakdown of host cell components, such as cell walls, proteins, and lipids. Overall, *F. graminearum* effectors are essential for the fungus's survival and virulence, thereby making them a critical component of *F. graminearum* pathogenesis, enabling the fungus to successfully establish an infection and cause disease. Detailed characterization of these effectors by gene expression, bioassay, and RNAi mediated silencing will enhance our understanding of this pathogen's biology and offer insights into new strategies for disease control.

1.8 Rationale and Aim of the Study

Fungi secrete effectors that play a pivotal role in infection by targeting a broad spectrum of plant genes, including those involved in signal transduction, metabolic pathways, and the plant's immune response. Previous studies have demonstrated that *F. graminearum* employs diverse effectors to cause infection in wheat (Lysoe et al., 2011). With this rationale, an attempt is made to explore the effectome of the Indian isolate of *F. graminearum*, TN01 isolated in this study.

1.9 Hypothesis and Aim of the study

We aim at identifying and analyzing *F. graminearum* strain TN01 to assess its pathogenicity and virulence in wheat and the model plant, *Arabidopsis thaliana*. We hypothesize that fungal effectors might possess a role in pathogenicity and virulence and induce defense responses. To prove our hypothesis, the following objectives were set up to investigate the host's reactions to *F. graminearum* TN01 in both compatible and incompatible interactions.

Objectives of the study:

- 1. Genome-wide search to identify putative effectors with a role in pathogenicity and virulence in *F. graminearum*.**
- 2. RNAi-mediated silencing of the candidate effector gene for virulence function in *F. graminearum*.**
- 3. Comparing compatible and incompatible wheat-*F. graminearum* interaction for differentially expressed metabolites and validation by gene expression studies.**

Chapter. 2

Genome-wide search to identify putative effectors with a role in pathogenicity and virulence in *F. graminearum*.

2.1 INTRODUCTION

The fungal pathogen, *F. graminearum* infects the spikes at the anthesis stage in wheat and barley. FHB disease is a serious concern in wheat and other cereal crops worldwide (Zhu et al., 2019). The fungal infection spreads along the length of the spike, thereby causing bleached spikes with shrivelled grains contaminated with mycotoxins. The fungus also secretes proteins that may function as virulence factors to promote pathogenesis in the host (Goswami & Kistler, 2004). The small secretory proteins, which are cysteine-rich and lack the transmembrane domain, are generically known as ‘effectors’. These effectors have diverse roles and are involved in suppressing host immune responses by manipulating host defense machinery (Gorash et al., 2021). The effectors are typically either extracellular (apoplastic), which carry signal peptide sequences, or intracellular (cytosolic), which are localized within the host cells. Studies have demonstrated that effectors in fungi with different lifestyles interact differently with their hosts (Lo Presti et al., 2015). Additionally, since *F. graminearum* predominantly targets floral tissues, the release of effectors might be specific to these tissues. Consequently, the timing of infection and the type of tissue involved could influence both the quantity and variety of effectors released into the host, and these factors should be considered. *F. graminearum* has been found to secrete a multitude of secretory proteins that are responsible for initiating the infection. However, as many as 600 hundred effectors were predicted by computational studies in *F. graminearum* (Brown et al., 2012). However, these effectors are yet to be characterized. Moreover, the predicted effector genes were upregulated in both asymptomatic and symptomatic tissues, suggesting their probable ubiquitous nature (Brown et al., 2017).

Studies carried out to unravel the mechanism of virulence of the *Fusarium* pathogen were primarily focused on understanding the potential Trichothecene mycotoxin-producing genetic components in other fungal members of the genus, *Fusarium*. The transcriptome studies carried

out to analyze *F. graminearum* infection in the host, revealed upregulation of several effector-coding genes, but they were predominantly hypothetical and unannotated (Lysøe et al., 2011). The poorly defined effector repertoire of the devastating *F. graminearum* pathogen necessitates not only detecting but also deciphering their role in pathogenicity and virulence in the host.

Therefore, the study aims at detecting and analyzing the putative effectors that are essential for pathogenesis and virulence which could be targeted to mitigate FHB or *Fusarium* head blight losses and mycotoxin contamination in crop plants. To identify the candidate genes encoding secretory effector proteins, a genome-wide *in-silico* search using computational tools was performed. To analyze their role in pathogenesis and virulence, gene expression studies during pathogenesis as well as bioassays for virulence were carried out in the model plant, *Arabidopsis*, and wheat and the findings are presented.

2.2 MATERIALS AND METHODS

2.2.1. Fungal isolation and culture conditions

The *F. graminearum* strain used in this study was isolated from the spikelets of wheat plants grown in field conditions at IARI Regional Station, Wellington, Tamil Nadu (11°22' °N longitude and 76°48' °E latitude located at an altitude of 1850 m above the sea level) at the temperature regime of 21 °C/31°Cday/night with 75% humidity. Single-spored cultures were grown on potato dextrose agar (PDA) for 7 days with alternating cycles of 12hour (h) light/12 h darkness at 25–28 °C. The purified single-spore culture of the fungus exhibited the typical salmon-colored mycelia and macroconidia formation characteristic of *F. graminearum*. The spores were confirmed by examining them with a light microscope (model: Leica; make Olympus-IX81) at 40x as shown in (Figure 2.1).

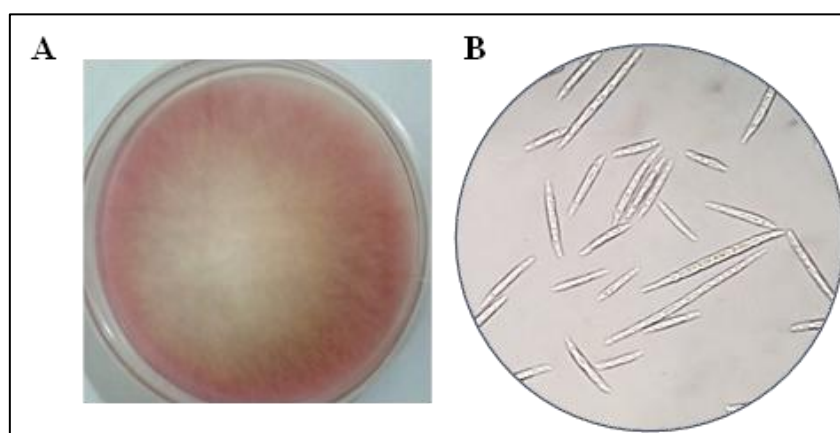


Figure 2.1 A single-spored fungal culture and macroconidia of *F. graminearum*. (A) The characteristic salmon-colored fungal culture at the sporulation stage grown on PDA. (B) Light microscopy image of macroconidia or fungal spores of *F. graminearum* viewed at 40x magnification.

To prepare the liquid culture, 4 g of mung bean seeds were washed and boiled for 5 min in 100 mL water and filtered before autoclaving at 121 °C at 15 psi for 15 min. About 1cm diameter mycelial plug was added to the cooled liquid medium. Cultures were kept for continuous shaking

at 150 rpm at 26 °C for 5 days. The macroconidia were passed through a 3-layered sterile cheesecloth and centrifuged for 10 min at 10,000 g. The pellet was washed with autoclaved water twice before resuspending in autoclaved double-distilled water and adjusted to a concentration of 10^5 conidia/ mL. To the spore solution, 0.05% (v/v) Tween 20 was added as a surfactant to disperse the spores in the solution, and the conidia concentration was adjusted to 10^5 conidia/ mL using a hemocytometer, and the spore suspension was stored at 4 °C.

2.2.2. Fungal DNA extraction

The fungal mycelium from the PDA plates was transferred to the mortar and pestle using a sterile spatula under aseptic conditions. The mycelia were pulverized in liquid nitrogen and the sample was homogenized in 2 mL of CTAB buffer. The CTAB- buffer contained 20 g/L CTAB, 1.4 M NaCl, 0.1 M tris-HCl, and 30 mM Na₂EDTA at pH 8.0. The ground sample was transferred to Eppendorf tubes and vortexed briefly followed by incubation at 65 °C for 30 min. 10 µL of RNase (10 mg/mL, Sigma) was added to the contents, and the samples were incubated at 37 °C for 30 min. Samples were centrifuged at 12,000 g for 10 min and the supernatant was transferred to a clean Eppendorf tube containing 500 µL of chloroform, vortexed, and centrifuged at 12,000 g for 15min. The supernatant was precipitated in 0.6 volumes of isopropanol overnight at -20 °C. The tubes were centrifuged for 10 min at 12,000 g, and the pellet was washed with 700 µL 70% ethanol, air-dried, and the pellet was re-suspended in 1x Tris-EDTA buffer (pH 8.0) (Mourelos et al., 2016). DNA was quantified by a Nanodrop spectrophotometer at 260 nm. The quality of the DNA was confirmed on 0.8% agarose gel. The DNA samples were diluted to working solutions of 10–25 ng/µL.

2.2.3. Plant material and growth conditions

The plant material comprised *Arabidopsis* (*Arabidopsis thaliana* L.) genotype Col-0 obtained from the Department of Plant Sciences, University of Hyderabad. The seeds were surface sterilized

with 0.01% sodium hypochlorite solution for 5 to 10 min and then washed with autoclaved water twice. Then seeds were subsequently washed with 70 % ethanol for 3 min, and finally, ethanol traces were removed using autoclaved water twice. Sterilized seeds were placed in peat soil and left at 4 °C for 3 days, and then transferred to a growth chamber and grown in pots at an optimum temperature of 22–23 °C for 16 h of light and 8 h of darkness, with a relative humidity of 50–65 %. After thinning the germinated seeds, one plant/pot was kept in the growth chamber continuously for 3 weeks with 20 h of light (13 °C)/4 h of dark (11 °C) and 40–60% relative humidity.

The two wheat genotypes, Nobeokabozu (Nobeoka) and Sonalika, which are resistant and susceptible to *F. graminearum* infection, respectively (Atanassov et al., 1994; Kumar et al., 2021), were sourced from the National Bureau of Plant Genetic Resources (NBPGR) in New Delhi. They were maintained and evaluated at the IARI Regional Station in Wellington, Tamil Nadu. Seeds were surface sterilized before germination on sterile plates to avoid mold contamination as previously described (Sorahinobar et al., 2016). The soil was autoclaved at 121°C for 30 minutes at 15 psi and allowed to cool for a day before planting. The seedlings were planted as three seedlings per pot with a diameter size of 7 inches. The plants were grown in a controlled growth chamber with 16 h light at 23°C and 8 hr dark at 20 °C with 50% relative humidity. Plants were watered at regular intervals on a daily basis and fertilized once a fortnight during the crop growth period.

2.2.4. Molecular and phylogenetic analyses of *F. graminearum* TN01

The fungal strain was identified through sequencing of the transcribed spacer (ITS) using ITS1/ITS4 (White et al., 1990), the D1-D2 region of the 28S region of nuc rDNA (D1-D2) using NL-1/NL-2 universal primers (Kwiatkowski et al., 2012) and translation elongation factor 1-alpha (*TEF1*) (Carbone & Kohn, 1999). A set of primers, ITS1 (5'-TCCGTAGGTGAACCTTGCGG-

3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the transcribed spacer (ITS) region while the primer set, NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-2 (5'-GGTCCGTGTTTCAAGACGG-3') were used to amplify D1-D2 region of the 28S region of nuc rDNA (D1-D2), and EF1 (5'-CATCGAGAAGTTCGA GAAGG-3') and EF2 (5'-TACTTGAAGGAACCCTTACC-3') were used to amplify (*TEF1*) gene. Amplified products were visualized on 1.2% agarose gels (**Figure 2.2**), stained with ethidium bromide and photographed under UV light in the Bio-Imaging system (DNR Bioimaging System, Israel).

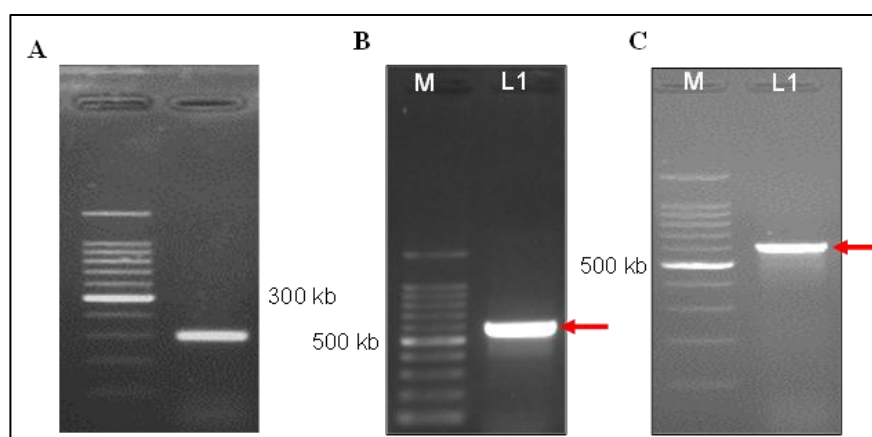


Figure 2.2 Representative gel images showing PCR-based amplification of (A) *TEF1α* gene, (B) ITS, and (C) D1-D2 region of ribosomal DNA (rDNA) of *F. graminearum* strain TN01. Lane-1 (L1) of the two gels (A) (B), and (C) show PCR products of *TEF1α*, ITS, and D1-D2, respectively, with band sizes ~300 and ~500-640 bp. M: 100 bp DNA ladder.

The PCR products were eluted from the agarose gel, purified, and confirmed by sequencing (**Table 2.2**). The sequences were submitted to the National Center for Biotechnology Information (NCBI). The evolutionary significance was inferred using the identified transcribed spacer (ITS), D1-D2 region of the 28S region of the nuc rDNA, and *TEF1*, gene sequences along with other reported nucleotide sequences including the *F. graminearum* strain PH-1 by the neighbor-joining method. The fungal strains of *C. cladosporioides* Gp01 were included to represent an out-group. The bootstrap (1000 replicates) values were computed (Kumar et al., 2016).

2.2.5. Genome-wide detection of Effector and non-effector gene candidates

The *F. graminearum* PH-1 genome sequence (Accession: PRJNA13839) with gene prediction and annotation available in the NCBI database (<https://www.ncbi.nlm.nih.gov/>) was screened for candidate effector genes (Brown et al., 2012; Lu & Edwards, 2016). The N-terminal signal peptides were predicted using SignalP 4.0. (<http://www.cbs.dtu.dk/services/SignalP/>). The highly conserved domains were predicted using the program Conserved Domain Database (CDD). The effector probability for each of the secreted proteins was further evaluated using EffectorP.3 (<http://effectorp.csiro.au/>) (Sperschneider & Dodds, 2021). The potential localization in plant cells for these putative genes was predicted by Wolf pSORT (<https://www.psort.org/>).

2.2.6. *In-silico* analysis of protein domains, motifs, 3D- structure, and protein-protein interaction

The MEME 5.5.3 (<https://www.meme-suite.org/meme/tools/meme>) motif search tool was used to identify candidate motifs and domains for the candidate effector and non-effector proteins (Bailey et al., 2009). The protein-protein interaction network of effectors was generated using a STRING database version 11.5 (<https://string-db.org/>). The protein structure of all candidate-secreted proteins was predicted with template-free modeling by TrRosetta. The colors of protein structural models are representative of the degree of conservation: red- a high level of conservation; blue- a low level of conservation. TrRosetta (Du et al., 2021) was run with the filtered MSAs to predict inter-residue orientations and distances, and full-atom models of the hypothetical effectors with the TrRosetta bioinformatics tool (<https://yanglab.nankai.edu.cn/trRosetta/>).

2.2.7. Primer designing

The oligonucleotide primers were designed from the coding sequence (CDS) of hypothetical effector and non-effector genes from the *F. graminearum* PH-1 genome. The primer-BLAST tool (<https://www.blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to design and test primer specificity of the primers for genomic, reverse transcriptase (RT), and real-time-quantitative (qRT-PCR). Oligonucleotides with their predicted amplicon size and annealing temperatures are listed in (Table 2.1).

2.2.8. PCR amplification and sequencing of putative effector and non-effector genes

The PCR reaction mix contained 1X master mix (TAKARA, JAPAN), 0.25 μ M of each primer, 1:10 diluted cDNA, and nuclease-free water to make up the 10 μ L final volume. The cycling conditions were standardized as initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing temperature between 50 and 60 °C for 35 s, extension at 72 °C for 35 s, and final extension steps at 72 °C for 5 min followed by a hold at 4 °C. PCR products were eluted from the gel by using NucleoSpin gel and a PCR clean-up kit (Macherey Nagel) and sequenced. Amplified products were then confirmed through a sequence similarity check using NCBI-BLAST as shown in (Table 2.5).

Table 2. 1 The list of primers of putative effector genes used for RT-PCR and qRT-PCR in this study along with primer sequences and annealing temperature.

S. No	Gene ID	Primer sequence 5'-3'	Amp Size (bp)	Annealing Temp (°C)
1	FGSG_00060*	F: ATGTATACTCTCCTCACTCTCACCA R: TCGCCATCGCAGCAAGGGTTCTCAA	386	60
2	FGSG_00061*	F: ATGCATTACTCTACTCTTACTACC R: TTAACAAGCTCCCTGGCAACTA	379	55
3	FGSG_00062*	F: ATGCACTTCTCAACATCAATTCTT R: CTAACAATGACAATTCCCCTCACA	402	56
4	FGSG_03599*	F: ATGCAGTTCACCACCTCCTT R: CACATCTAAGCACAGGCAGT	294	55
5	FGSG_11205*	F: ATGCAGCTGACCAACCTCTTCTGT R: TCAATCCACAGTTGCTAACAGCGA	415	51
6	FGSG_07921	F: TGCTACACAGACACCGCTTT R: GCTCGAACCTTGGAGGGATT	224	53
7	FGSG_03748	F: GTTACATCTCCCTGCCACC R: AGCTCAACCAAGTGTCCGTT	241	53
8	FGSG_03581	F: TGCTGCCGACTTTACCAACT R: TCCGTTCTTGCTGTTCTGCT	151	53
9	FGSG_05341	F: TGGTTGGCGATGAGGACATT R: GCCAGTTGACTCAATCCCGT	161	53
10	FGSG_04213	F: CAGAAGAGCACAAACACCGC R: ACTTGAAGGTGGCAGAGTCG	166	53
11	FGSG_10206	F: GCCACTGCTGCTTCACTCTA R: GTCGACGCATTTAACCTCGC	197	53
12	FGSG_10603	F: CCTTCCACTACACAAGCGGT R: TTGCATTGCGCTTTGTTCCA	192	55
13	FGSG_00444	F: CCGCCTTTGATCACTCACACT R: TATCGGAAAGCGACGAGAGC	199	55
14	FGSG_13281	F: TAGTAGTGGAGGTGGTGGCT R: ATCATCGTCATCCCAAGCCT	310	53
15	FGSG_09127	F: ATGCAGATCTTCACTCTCGC R: CTAGTTAGCCTCAGCTCCCT	348	53
16	FGSG_09908	F: ACTTTCCGYCGCATCYTSAT R: CAATCTTKGATCGCTCGTATG	116	55
17	FGSG_03537(<i>TRI5</i>)	F: TCTTAACACTAGCGTGCGCCTTCT R: CATGCCAACGATTGTTTGAGGGA	100	59
18	FGSG_16251(<i>TRI6</i>)	F: TAACCACATCGTCGGGACTG R: GCCGACTTCTTGACAGGTCTT	110	53
19	<i>β-Tubulin</i>	F: ATCTCGAGCCCGGTACCATGG R: CTCGGTGTAATGACCCTTGCC	200	57
20	<i>GAPDH</i>	F: TGACTTGACTGTTGCGCTCGAGAA R: ATGGAGGAGTTGGTGTGCGGTTA	200	57
21	<i>ACT8</i>	F: CTCAGGTATTGCAGACCGTATGAG R: CTGGACCTGCTTCATCACTCTG	160	62

* Oligonucleotide primer sequences (Lu and Edwards, 2016).

2.2.9. Detached leaf assays in Arabidopsis

Four-week-old *Arabidopsis* plants had their leaves treated with 10 µL of 10^5 conidia/mL *F. graminearum* on the upper surface using a syringe without a needle. The leaves were then placed on damp absorbent cotton inside petri dishes and samples were observed under a light microscope at intervals of 0, 12, 24, 48, and 72 hours after inoculation (hpi). Leaves treated with autoclaved sterile water were used as a control group.

2.2.10. Fungal Inoculation

At the mid-anthesis stage, the middle spikelets 4th or 5th from the base of the spike of Sonalika and Nobeoka genotypes were point-inoculated by placing 10 µL of 10^5 /mL conidia per spikelet (Feng et al., 2018). The mock-treated samples with water served as controls. The inoculated and control spikes were maintained at 25 °C and covered with zip-lock bags to provide high relative humidity for *F. graminearum* infection and removed after 48 hpi. Autoclaved water was used for the control treatment. Samples were collected for all the time points for the RNA extraction (**Figure 2.3**).

2.2.11. Detached leaf assays on wheat

For the detached leaf assay, 7-day-old plants of both wheat genotypes were grown in a hydroponic plant growth medium made up of Hoagland solution. The Hoagland solution was prepared as given by (Mathivanan & Raghupathi, 2017). Three to five cm leaf segments were excised and placed on petri plates containing water agar for 3 days. The leaves were inoculated with *F. graminearum* TN01 conidia suspension prepared as described above under aseptic conditions. A 10 µL spore suspension of concentration, 10^5 /mL conidia were placed on the adaxial surface of wheat leaves while the control leaves were mock-inoculated with autoclaved distilled water. The leaf segments were incubated in a laboratory incubator at a low temperature (10 ± 1 °C) under continuous white light at room temperature (21 ± 2 °C) with three replicates (Petri dishes) per treatment (Brown & Cook., 2004).

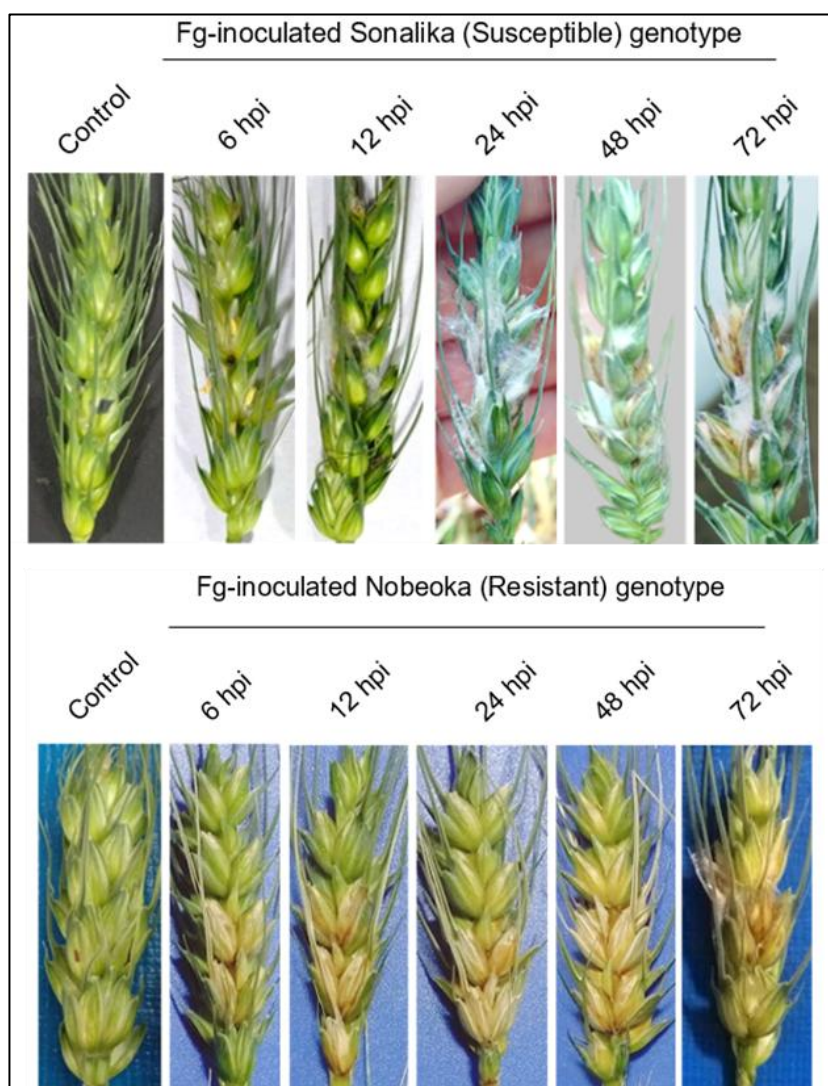


Figure 2.3 Representative images of Wheat spikes collected at different time points post *F. graminearum* (*Fg*)-inoculation.

2.2.12. RNA extraction and cDNA synthesis

Total RNA was extracted from the leaves of four-week-old *Arabidopsis* plants. In wheat, total RNA was isolated from 5-6 *F. graminearum*-inoculated spikelets of the same age per genotype for each time point. The samples were harvested at 0, 6, 12, 24, 48, and 72 hpi. The *F. graminearum*-inoculated and mock (water)-treated wheat spikelets were lyophilized and homogenized for RNA isolation and cDNA synthesis using the company-manufactured TRIZOL

(RNAiso Plus, TAKARA, Japan) following the manufacturer's instructions. The tissue samples were ground in liquid nitrogen using a pre-chilled mortar and pestle further, homogenized in 1 mL of Trizol, and incubated for 5 min at room temperature. Centrifugation at 13,000 g for around 15 min at 4 °C was performed. In a fresh nuclease-free Eppendorf tube, an aqueous phase was collected and 500 μ L of isopropanol was added /mL of TRI- reagent for the precipitation step. The Eppendorf tubes were incubated for 30 min at -80 °C and then centrifuged again at 12000 g for 15 min. The pellet was washed with 75 % ethanol (w/v) and finally, the RNA pellet was dried and resuspended in the nuclease-free water. Three biological replicates for each time point were analyzed per treatment (Hao et al., 2020). First-strand cDNA obtained from 1 μ g of total RNA was synthesized using the cDNA synthesis kit from (TAKARA, Japan). Using the cDNA as a template, PCR optimization conditions were standardized for the candidate effector and non-effector gene amplification.

2.2.13. Differential expression analyses of effector and non-effector genes by RT- PCR

Using the cDNAs as templates, PCR amplification efficiency, and optimization conditions were standardized for the candidate effectors. The RT-PCR conditions were the same as described above (the PCR amplification and sequencing of putative effector and non-effector genes section) in a total reaction volume of 10 μ L. Reactions were performed in a thermal cycler (BioRad, USA) using the following PCR conditions: Initial denaturation at 95 °C for 5 min, 35 cycles of final denaturation at 94 °C for 35 s, annealing at 56 °C for 35 s, extension at 72 °C for 35 s, final extension at 72 °C for 7 min, followed by cooling at 4 °C. The amplicons were eluted from agarose gel using NucleoSpin Gel and a PCR clean-up kit (Macherey Nagel) and confirmed by sequencing (**Table 2.5**).

2.2.14. qRT-PCR-based expression analyses of effector and non-effector genes

The real-time quantitative polymerase chain reaction (qRT-PCR) was carried out in a 96-well PCR plate from (Applied Biosystems). The PCR efficiency of these primers was evaluated using the varying template concentrations of the cDNA dilution as 1:5, 1:10, and 1:20. The primer concentration of 0.25 μ M was used. The 10 μ L reaction mixture was composed of 5.2 μ L of 1X SYBR mix using (TB Green® Premix Ex Taq™ II, TAKARA), 0.5 μ L of 0.25 μ M primers, 1 μ L of 1:5 diluted cDNA, and 2.8 μ L of water. The cycling conditions included an initial denaturation of 95 °C for 5 min, followed by 35 cycles at 95 °C for 35 s, a specified annealing temperature for a specific gene for 30 s, 72 °C for 35 s, followed by 60 °C for 15 s. All the samples including the non-template DNA control were set in triplicate (NTC). The real plex program (Applied Biosystem) was used to calculate the threshold cycle value (Ct) for each sample for both effector and housekeeping genes. Gene-specific primers used for each of the effector genes are listed (Table 2.1). The relative expression level of each gene was normalized by the constitutively expressed β -*Tubulin* gene and was calculated based on the $2^{-\Delta\Delta C_t}$ values (Livak & Schmittgen., 2001).

2.2.15. Microscopic investigation for pathogen growth

Evaluation of the infection at the microscopic level was important to carry out by performing time course infection studies. The leaves of *F. graminearum*-inoculated and control samples of Arabidopsis and wheat genotypes were stained with trypan blue to monitor the fungal infection. The trypan blue stock solution was made by combining 0.02 g of trypan blue dye with 10 g phenol, 10 mL glycerol, and 10 mL lactic acid in 10 mL sterile distilled water. The working solution was made by diluting the stock solution in 1:2 (v/v) ratios with ethanol (96%). To remove chlorophyll, the samples were treated with 3:1 ethanol: glacial acetic acid for bleaching overnight. Leaf samples were treated with trypan blue for 1 hour followed by de-staining with lacto-glycerol solution

(equal volumes of lactic acid, glycerol, and water in a 3:1:1 ratio). Trypan blue-stained *F. graminearum* (Fg)-inoculated leaf samples were mounted on clean glass slides using 60% glycerol and examined under a light microscope (Bhosle et al., 2019).

2.2.16. Localization of H₂O₂ in leaves

The Localization of Hydrogen peroxide (H₂O₂) in *F. graminearum*-inoculated leaves at different time points in both genotypes was performed using DAB (3, 3'-diaminobenzidine) staining, followed as previously described (Bhosle et al., 2019) in Arabidopsis. For wheat, 4 cm-long mid-section leaf segments were taken from 2-week-old wheat plants. Each leaf segment was placed with the adaxial surface facing up over a moistened sterile cotton placed in a petri plate for 7 days. The samples were de-stained by treating with bleaching solution (ethanol: acetic acid: glycerol in 3:1:1) at 95 °C for 15 min to clear the chlorophyll content. The leaf samples were transferred to a fresh bleaching solution for 30 min. The samples were examined using a light microscope. Images were taken at 40x magnification.

2.2.17. Fusarium head blight (FHB) pathogenicity assay

Fusarium graminearum-inoculated wheat heads were examined for FHB disease incidence such as bleached spikelets seen on the ears of both resistant and susceptible genotypes studied at different time points after inoculation. At 7 dpi, disease symptoms occurred on the inoculated spikelets. The FHB severity was calculated in both cultivars at 7 and 10 dpi. Mock-treated spikes served as control. The percentage of infected spikelets was calculated using 3 replicates with 3 plants each. The experiments were repeated twice to check for the reproducibility of the findings.

2.3 RESULTS

2.3.1 Morphological, molecular, and phylogenetic analysis of *F. graminearum* TN01

The fungal strain TN01, isolated from wheat spikes, was confirmed as *F. graminearum* through both morphological and molecular analyses, focusing on the internal transcribed spacer (ITS), the D1-D2 region of the 28S nuclear rDNA, and the *TEF1* gene (**Figure 2.2**). PCR amplification of the ITS and D1-D2 regions of the 28S nuclear rDNA yielded DNA products ranging from 500 to 640 base pairs, while the *TEF1* gene produced approximately 300 base pairs (**Figure 2.2**). These sequences were submitted to NCBI with GenBank accession numbers OR073525.1 for ITS, MK814342.1 for the D1-D2 region, and PP726712 for the *TEF1*-alpha gene. BLAST search results for the ITS, D1-D2 region, and *TEF1* of TN01 showed significant nucleotide similarity with known *F. graminearum* strains, with 99.46% and 99.83% similarity for the ITS and D1-D2 regions, respectively, compared to accession numbers OQ438114 for ITS and MH8773561 for the D1-D2 region in NCBI as shown described in (**Table 2.2**).

Table 2.2. List of Sequences used for confirmation of the *F. graminearum* isolate, TN01 using universal identification primers.

Region name	Accession No.	Query cover	% Identity	Gene Bank Accession
Internal transcribed spacer (ITS)	OR073525.1	98 %	99.46%	OQ422503
28S region of nuc rDNA (D1-D2)	MK814342.1	99 %	99.83%	MH877275
Translation elongation factor 1-alpha (<i>TEF1</i>)	PP726712	98 %	99.61 %	OM650303

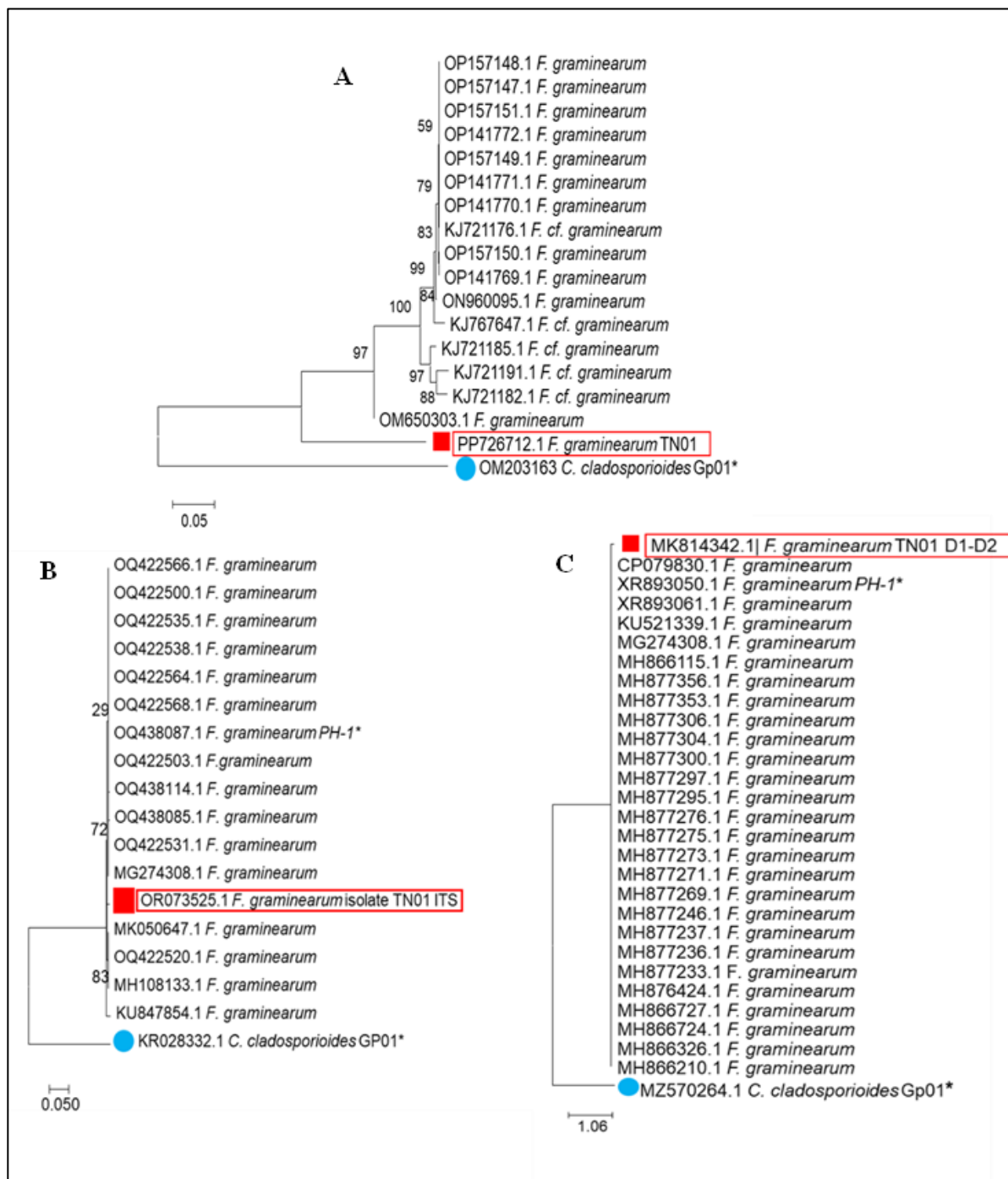


Figure 2.4 The phylogenetic relationship was inferred based on (A) *TEF1α*, (B) ITS1, and (C) D1-D2 region of the large subunit of ribosomal DNA by the Neighbor-Joining method by using MEGA 11. The *F. graminearum* strain TN01 used in the study is highlighted in red color. The outgroup fungal strains, *C. cladosporioides* Gp01 included in *TEF1α*, ITS, and D1-D2 dendrograms respectively are indicated in blue color with an asterisk mark.

Additionally, the phylogenetic relationship of TN01 was evaluated against previously reported *F. graminearum* strains, including the *F. graminearum* strain PH-1, using the neighbor-joining method with the optimal model and 1000 bootstrap replicates (Tamura et al., 2021). Dendrograms were constructed for the *TEF1* (**Figure 2.4 A**), ITS (**Figure 2.4 B**), and D1-D2 region (**Figure 2.4 C**) of TN01, showing that all *F. graminearum* strains, including TN01, clustered into a single clade, indicating high similarity. In contrast, *C. cladosporioides* Gp01, used as an outgroup for the *TEF1*, ITS, and D1-D2 region, phylogenetic trees, formed a separate group.

2.3.2. *In-silico* based Genome-wide screening

Genome-wide *in-silico* search for putative effector and non-effector genes was carried out by downloading all the proteins predicted in the complete genome sequence of *Fusarium graminearum* PH-1. The *in-silico* analysis of the polypeptide sequences of length <300 aa without transmembrane domains led to the detection of approximately 309 sequences (**Figure 2.5 A**). Of these 309 sequences, a total of 290 putative effectors were retrieved with 243 cytoplasmic and 47 apoplastic effectors. Out of the 309 sequences, 98 gene sequences showed predicted protein family domains (Pfam) suggesting that these were potential candidate effectors (**Figure 2.5 B-C**). The percentage of effectors with a high prediction value (>0.9) was 7%, whereas those with a prediction value between 0.7 and 0.89 were 25%. Likewise, of the total sequences, the sequences with predicted signal peptides were 14%. Among these, 16 effector and non-effector gene sequences with high prediction values and signal peptide-containing sequences were tested to analyze their role in virulence and pathogenesis. The selected putative effector and non-effector sequences showed N-terminal signal peptides but lacked transmembrane domains. The other possible characteristics like signal peptides, functional domain, and predicted function of these effectors are listed in the (**Table 2.3**).

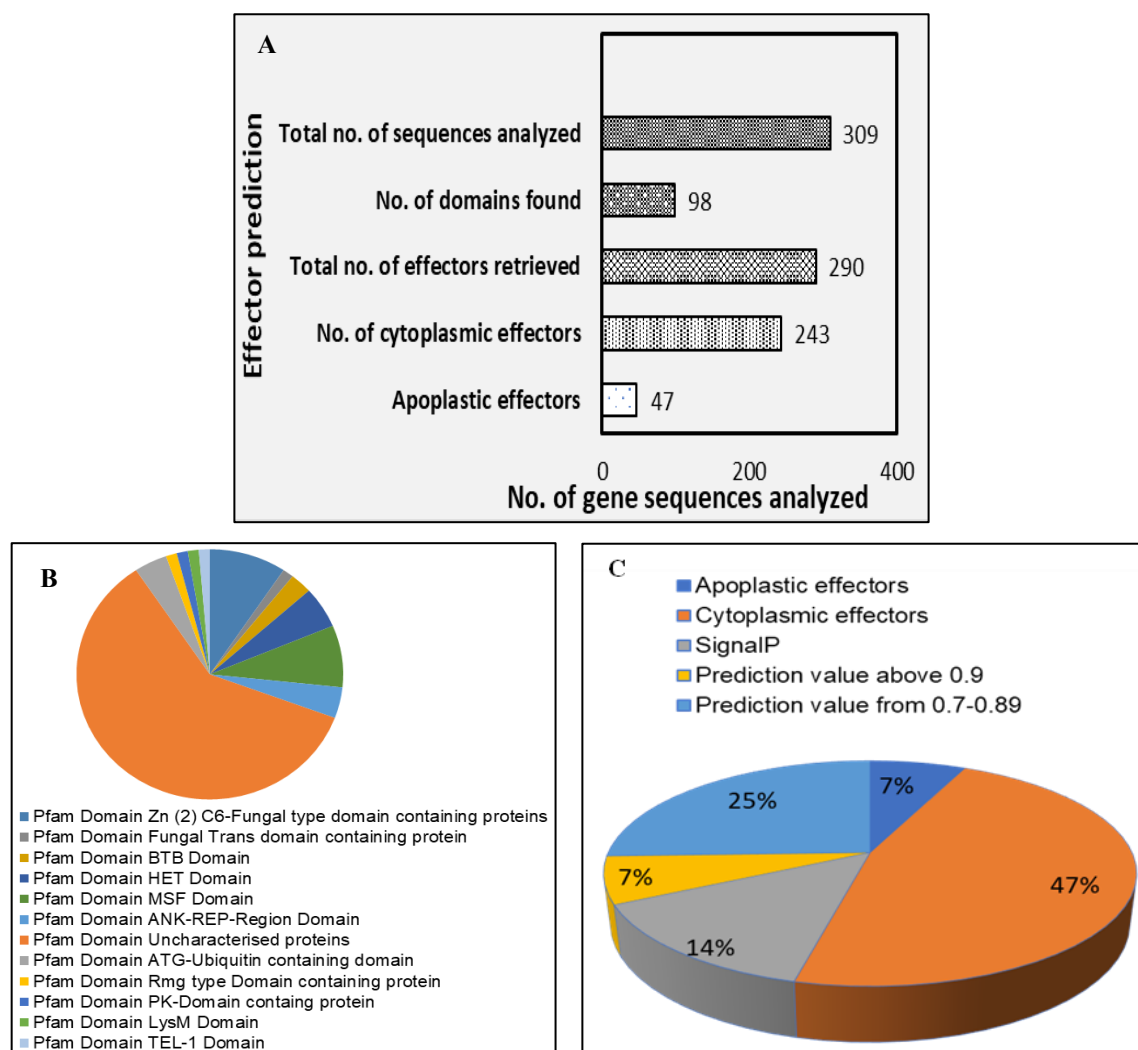


Figure 2.5 Genome-wide *in silico* search of putative effector genes in *F. graminearum* strain PH-1. Graphical representation of (A) total no of protein sequences with predicted effectors; (B) total number of predicted Pfam domains of effectors grouped based on their function and percent signal peptide sequences; (C) Percentage of predicted effectors along with the value.

The putative effector gene, FGSG_13281 though a hypothetical gene, showed the presence of ZnF_GATA domain while FGSG_11205 showed Pfam07249 with ceratoplatanin functional domain involved in the necrosis-inducing activity on the host. FGSG_07921 had the characteristic DOMON-like ligand binding domain having a heme-binding motif with cellobiose dehydrogenase (CBM9_like 3) activity while FGSG_03748 showed TNT-domain having ribonuclease-toxin activity that is involved in the necrosis induction in the infected cell and the defense mechanism. Another candidate effector, FGSG_05341, showed homology with the *Cladosporium fulvum*

Ecp2, a pathogenesis factor, while the remaining candidate effector genes were shown as hypothetical effectors.

Table 2.3. Characteristics of 16 putative effector and non-effector genes of *Fusarium graminearum* detected through *in-silico* and gene expression studies in Arabidopsis and wheat.

Gene Id	Ch. Location	Size (aa)	#Cys	SignalP (aa)	Predicted function	Annotation EffectorP.3	Pfam domain
FGSG_00060	1	134	11	17	KP4 killer toxin*	Effector	Pfam09044
FGSG_00061	1	125	9	19	KP4 killer toxin	Effector	Pfam09044
FGSG_00062	1	133	11	17	KP4 killer toxin	Effector	Pfam09044
FGSG_03599	2	95	11	19	CFEM*	Effector	NA
FGSG_11205	3	140	6	19	Ceratoplatanin*	Effector	pfam07249
FGSG_07921	4	221	5	16	Hypothetical	Effector	cd09620
FGSG_03748	2	253	5	18	Hypothetical	Effector	pfam14021
FGSG_03581	2	198	5	21	Hypothetical	Effector	NA
FGSG_05341	3	189	6	17	Hypothetical	Effector	pfam14856
FGSG_04213	2	166	6	18	Hypothetical	Effector	pfam16541
FGSG_10206	1	162	10	17	Hypothetical	Effector	NA
FGSG_10603	1	158	10	22	Hypothetical	Effector	NA
FGSG_00444	1	286	3	NA	Apoptosis-linked gene 2	Effector	cd16180
FGSG_13281	2	189	3	NA	Hypothetical	Effector	ZnF_GATA
FGSG_09127	4	115	1	19	Hypothetical	Effector	cl00473
FGSG_09908	1	399	2	NA	cAMP regulatory protein	N/E	cd00038

The length of the amino acid sequences varied among all the effector genes (FGSG_03599). Based on the chromosomal location, 7 candidate genes were located on chromosome-1, 5 effector genes were located on chromosome-2, 2 effector genes were located on chromosome-3 and the other 2 effector genes were located on chromosome- 4. The cysteine residues, a characteristic of the effector genes also varied among the effector gene sequences. A higher number of cysteine

residues were detected in putative KP4 killer toxin genes with 11 residues in FGSG_00060, 9 residues in FGSG_00061, and 11 residues in FGSG_00062. Likewise, the FGSG_03599 (CFEM; common in fungal extracellular membrane) gene also displayed 11 cysteine residues indicating it as a potential effector.

The MEME suite (Multiple Expectation Maximizations for Motif Elicitation) was used to analyze the motifs of fungal effector proteins (Bailey et al., 2009). The number of theme identifications comprised a maximum of 10 numbers and the motif's occurrence was set to any number of iterations. The minimum and maximum motif widths were 3 and 50, respectively. All the 16 hypothetical effector gene sequences showed high consensus motif sequences while no motifs were observed for the two genes as shown in **(Figure 2.6)**. The identified motif sequences were also found in other *F. graminearum* genes as well when analyzed with the help of Motif Alignment and Search Tool (MAST). This tool enabled sequence search for matches using the best-combined match to all the motifs. The consensus motif sequences detected in the effector and non-effector genes of *F. graminearum* along with the characteristic conserved consensus motif sequence are presented in **(Supplementary Table 3)**.

In order to understand the possible roles of these effector candidates, the 3-D structural features of the putative effector and non-effector genes which comprise both annotated and hypothetical genes, were predicted by the template-free modeling tool, TrRosetta. These 3D structures provide information regarding the function of these unknown effector and non-effector proteins. Its evaluation is based on the importance of threading template layouts and the combination parameters of structure assembly simulations, which are typically in the range of 2 to 5. The TM-score, on the other hand, is a suggested scale for assessing the basic convergence/similarity of two structures. A TM-score of >0.5 suggests a valid topology model. A structure with a probability greater than 0.5 was deemed a reliable 3D structure as shown in **(Figure 2.7)**. The structure with

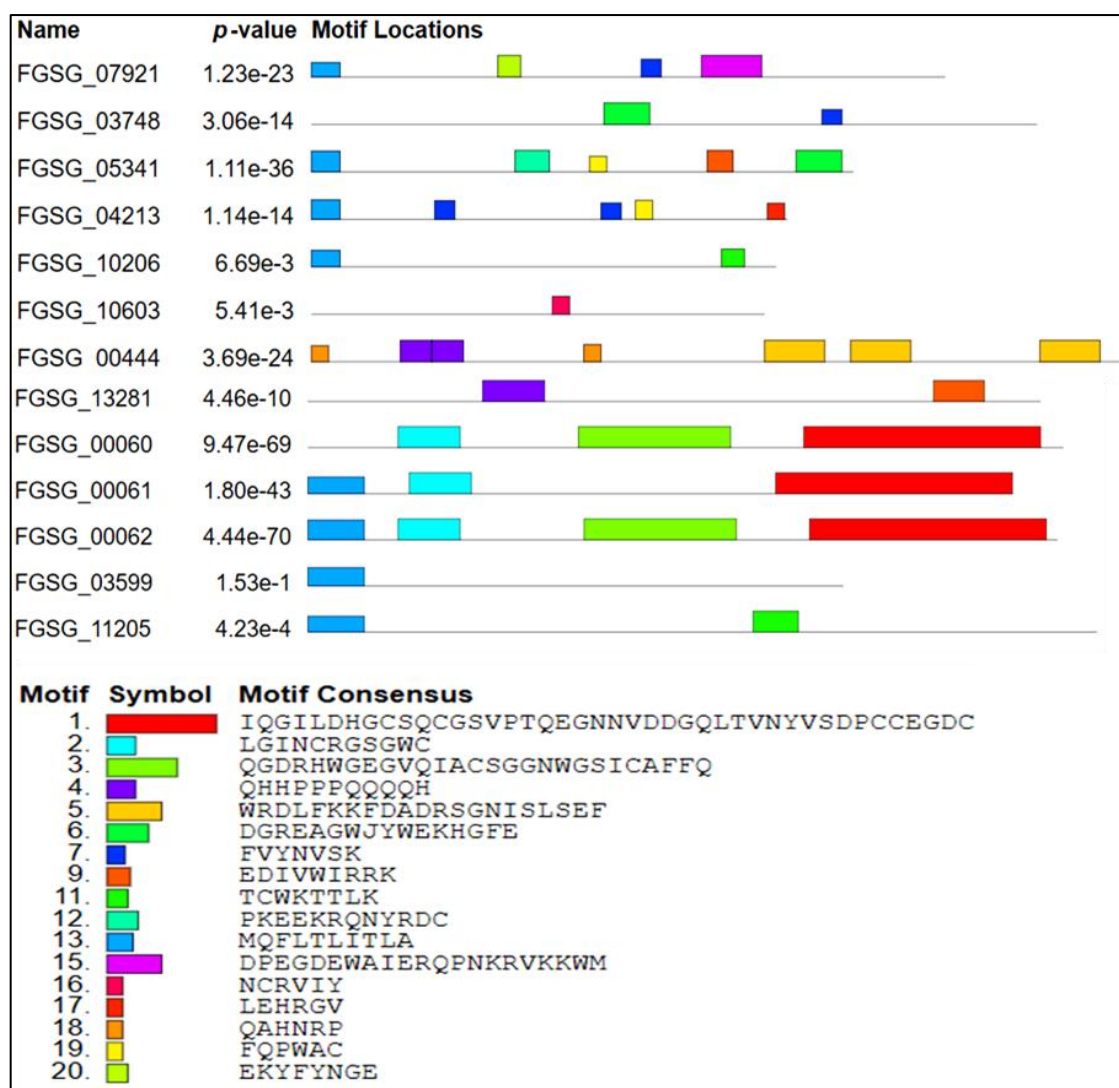


Figure 2.6 The conserved motifs sequences detected in the protein sequences of putative effector genes. Twenty distinct motifs, referred to as motifs 1–20, were found by using the full-length protein sequences of the selected putative effector genes using MEME.5.5.3 software.

the highest estimated accuracy among the TrRosetta outputs was chosen as the final model. Most of the effector structures were found to belong to the Hydrolase family. FGSG_03846 shows a similar match with the *Ustilago maydis* Hydrolase class of effector fold with 3ZPX as PDB ID. Also, this effector gene showed similarity with the cell wall degrading lipase A acting as the virulence factor in *Xanthomonas oryzae* with the PDB ID as 3H2G (Aparna et al., 2009) Another effector gene FGSG_13281 showed a strong hit with the PDB ID ZFHO *Saccharomyces cerevisiae* Ymr074 protein with unknown function. The putative effector, FGSG_00444, an EFF

hand domain protein is involved in the calcium-binding and signal transduction, whereas the other candidate genes– FGSG_10206, FGSG_05341, and FGSG_03581 did not show any hits from the PDB when analyzed by TrRosetta software (**Table 2.4**). Another gene, FGSG_09127 showed a hit with 61QC which represents the DNA binding protein involved in the programmed cell death in *Saccharolobus solfataricus* P2 gene. Interestingly, this effector gene showed another hit with the 2JXN (PBD structure) which is an uncharacterized PDCD5-like protein from *S. cerevisiae*. FGSG_03748 with PDB structure as 6YGE-B present in *Aspergillus fumigatus* (Stromland et al., 2021). Also, one of the effector genes showed a strong hit with 4AOG-A which is a structural protein belonging to the surface active Hydrophobin class II cluster showing the same core fold as observed in other class II Hydrophobins for example in *Verticillium dahlia* VDH1 protein (Klimes & Dobinson, 2006). FGSG_04213 showed homology with the PevD1 (*V. dahliae*) 5XMZ PDB structure, one of the signaling proteins responsible for hypersensitive response (HR) in tobacco.

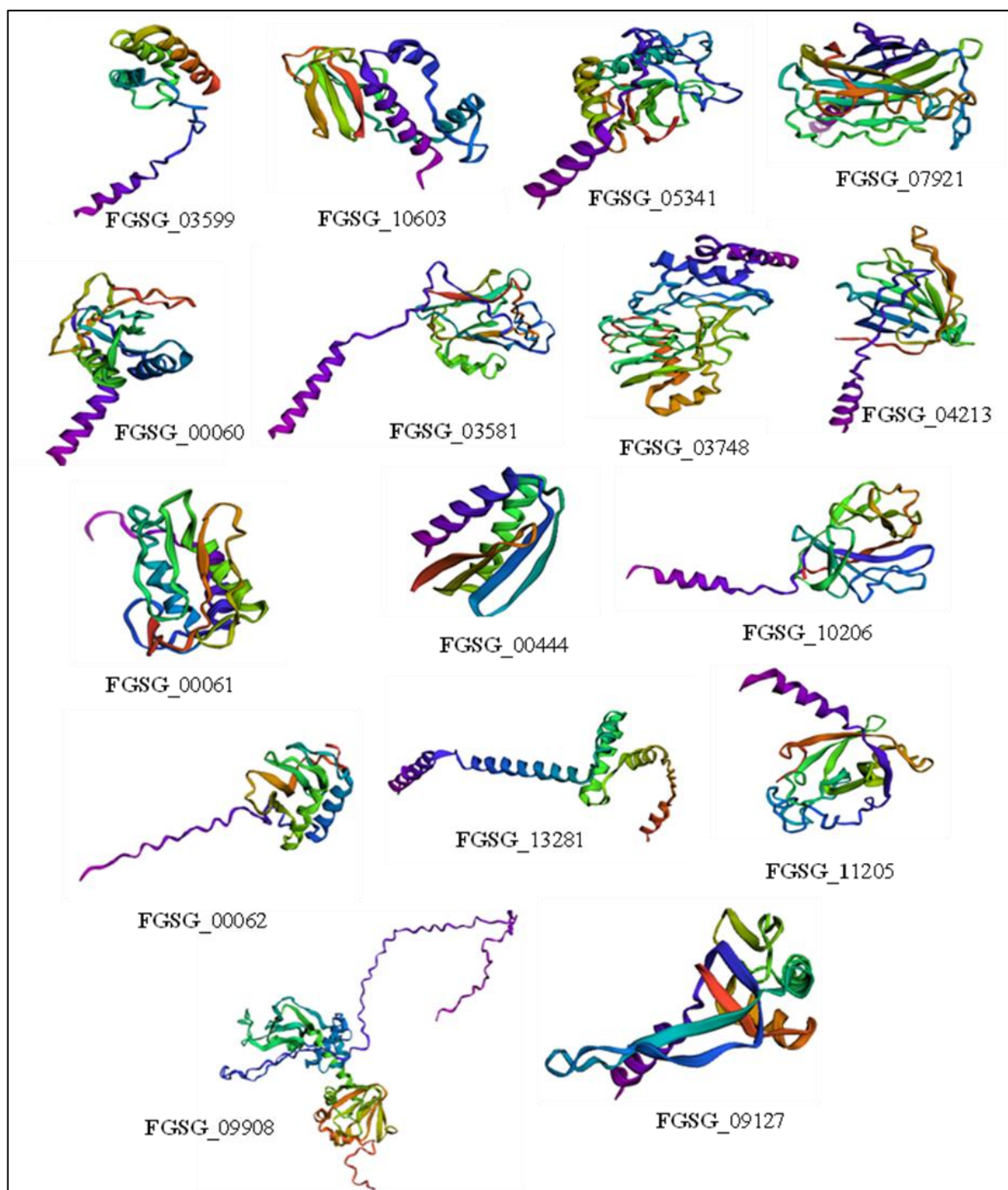


Figure 2.7 Putative effector and non-effector genes of *F. graminearum* with their 3D structure predicted by TrRosetta.

Table 2.4 Putative effector and non-effector genes of *F. graminearum* with their 3D structure prediction statistics using template-free modeling TrRosetta.

S. No	Gene ID	Annotation	TM Score ^a	Match ^b
1	FGSG_00060	KP4 toxin	0.431	1KPT
2	FGSG_00061	KP4 toxin	0.347	1KPT
3	FGSG_00062	KP4 toxin	0.875	1KPT_4
4	FGSG_03599	CFEM	0.605	---
5	FGSG_11205	Ceratoplatanin	0.640	---
6	FGSG_07921	Hypothetical protein	0.650	---
7	FGSG_03748	Hypothetical protein	0.880	6YGE
8	FGSG_03581	Hypothetical protein	0.873	---
9	FGSG_05341	Hypothetical protein	0.290	---
10	FGSG_04213	Hypothetical protein	0.867	5XMZ_A
11	FGSG_10206	Hypothetical protein	0.845	---
12	FGSG_10603	Hypothetical protein	0.418	---
13	FGSG_00444	Apoptosis-linked gene 2	0.607	---
14	FGSG_13281	Hypothetical protein	0.734	ZFHO
15	FGSG_09127	Hypothetical protein	0.744	61QC
16	FGSG_09908	cAMP regulatory protein	0.813	---

Abbreviations: KP4: Killer protein toxin, CFEM: Common in fungal extracellular membrane, TM: Template modelling.

2.3.3. Confirmation and expression studies of effector candidates

Genomic PCR with gene-specific primers confirmed the putative effector and non-effector genes in *F. graminearum* TN01. The fungal-specific β -Tubulin gene and non-template DNA control (NTC) served as positive and negative controls (**Supplementary Figure 1**). These putative candidate genes amplified a unique DNA band at the RT-PCR level. The gene sequences were confirmed by BLAST search using reported sequences as queries, showing >90% similarity and coverage with hypothetical genes in NCBI. Gene bank Accession numbers with query cover and % age identity are in the (**Table. 2.5**).

Table 2.5 BLAST results of effector and non-effector gene sequences of *F. graminearum*

S. No	Gene ID	Gene Bank Accession No.	Query cover	% Identity	mRNA Accession No.
1	FGSG_00060	PP883709	100%	99.26%	XM_011317353.1
2	FGSG_00061	PP909002	100%	100%	XM_011317354.1
3	FGSG_00062	PP909003	100%	100%	XM_011317355.1
4	FGSG_03599	PP889264	100%	100%	XM_011323804.1
5	FGSG_11205	PP909006	100%	100%	XM_011326834.1
6	FGSG_07921	PP909005	100%	100%	XM_011329456.1
7	FGSG_03748	PP909000	100%	100%	XM_011323641.1
8	FGSG_03581	PP889266	100%	99.83%	XM_011323822.1
9	FGSG_05341	PP889263	100%	100%	XM_011325559.1
10	FGSG_10206	PP909001	100%	100%	XM_011320851.1
11	FGSG_10603	PP909007	100%	100%	XM_011321304.1
12	FGSG_13281	PP909004	100%	99.75%	XM_011321542.1
13	FGSG_09127	PP889265	100%	99.43%	XM_011330359.1
14	FGSG_04213	PP889581	100%	100%	XM_011323110.1
15	FGSG_00444	PP889416	29%	100%	XM_011317811.1
16	FGSG_09908	XP_011318814.1	60%	100%	XM_011320512.1

2.3.4. Differential gene expression analysis in Arabidopsis and wheat leaves

To check the expression of these putative effector genes, gene expression studies were carried out by RT-PCR in the *F. graminearum*-inoculated leaf samples of Arabidopsis and wheat at a later time point of 48 hours post inoculation (hpi). The majority of the candidate genes expressed in both Arabidopsis and wheat leaf samples when challenged by *F. graminearum* as shown in (**Figure 2.8**). Of these 16 genes, the expression of 7 genes— FGSG_00060, FGSG_04213, FGSG_07921, FGSG_11205, FGS_03581, FGSG_10603 and FGSG_13281 were not observed in

Arabidopsis leaves. Likewise, all these 7 genes along with FGSG_00061 did not express in wheat leaves. The mRNA transcripts of the putative genes FGSG_09908 (non-effector) were higher in *F. graminearum*-inoculated Arabidopsis and wheat leaves which showed relatively reduced expression levels in *F. graminearum*-inoculated wheat leaf tissue compared to Arabidopsis at 48 hpi suggesting genotype-specific interaction.

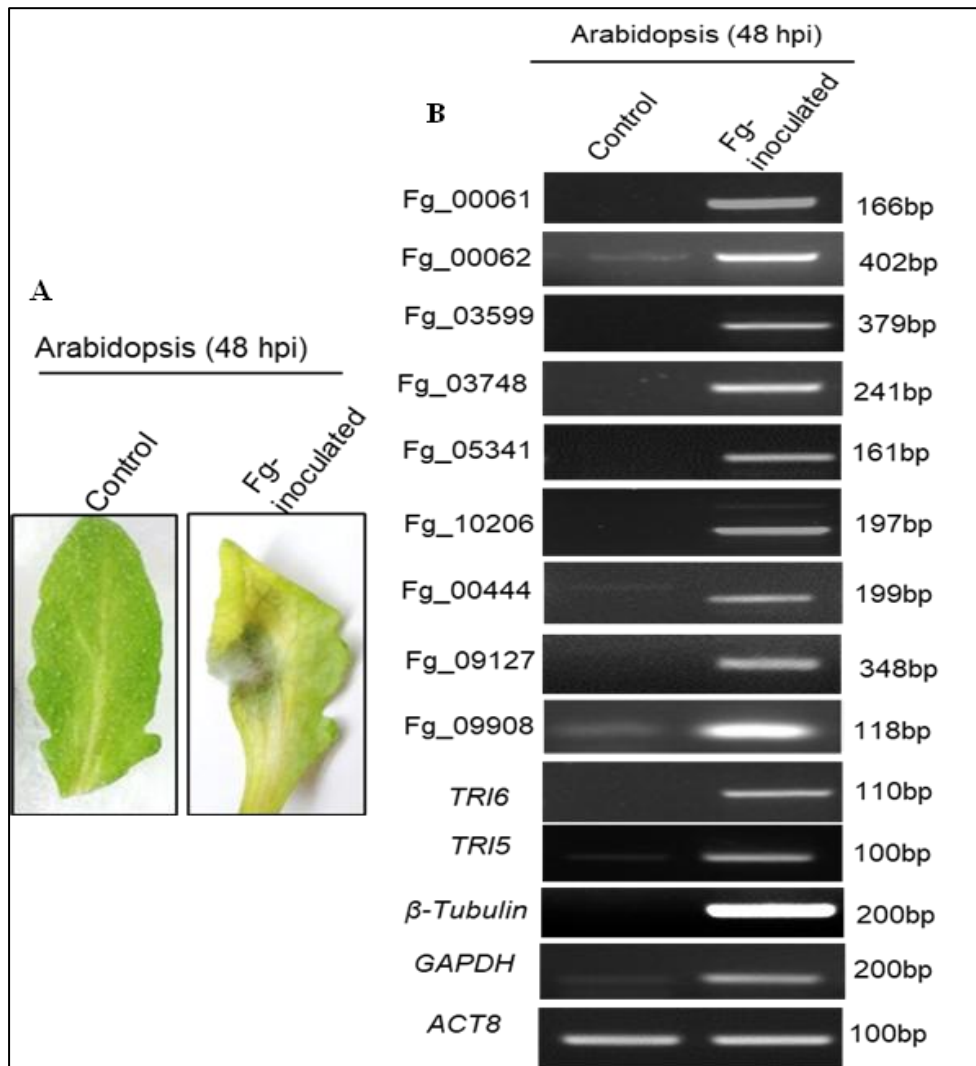


Figure 2.8 Reverse Transcriptase (RT-PCR) of the effector and non-effector gene candidates in *F. graminearum* (Fg)-inoculated Arabidopsis and wheat leaves compared to mock (water)-treated control at 48 hpi. (A) Disease phenotype and (B) candidate gene expression in *F. graminearum* (Fg)-inoculated Arabidopsis (wild type Col-0) leaves.

2.3.5. Differential effector and non-effector gene expression analysis in wheat leaves

One of the genes (FGSG_09908) was induced in the FHB susceptible genotype, Sonalika, in response to the pathogen but not in the FHB resistant genotype. Nobeoka suggested this as a putative pathogenicity factor. Two putative killer toxin genes— FGSG_00061 and FGSG_00062 belonging to the Kp4 superfamily were induced by *F. graminearum* in Arabidopsis, while the expression of FGSG_00062 alone was observed in *F. graminearum*-inoculated wheat leaves, suggesting the possibility of redundancy of these killer toxin genes as shown in (Figure 2.9).

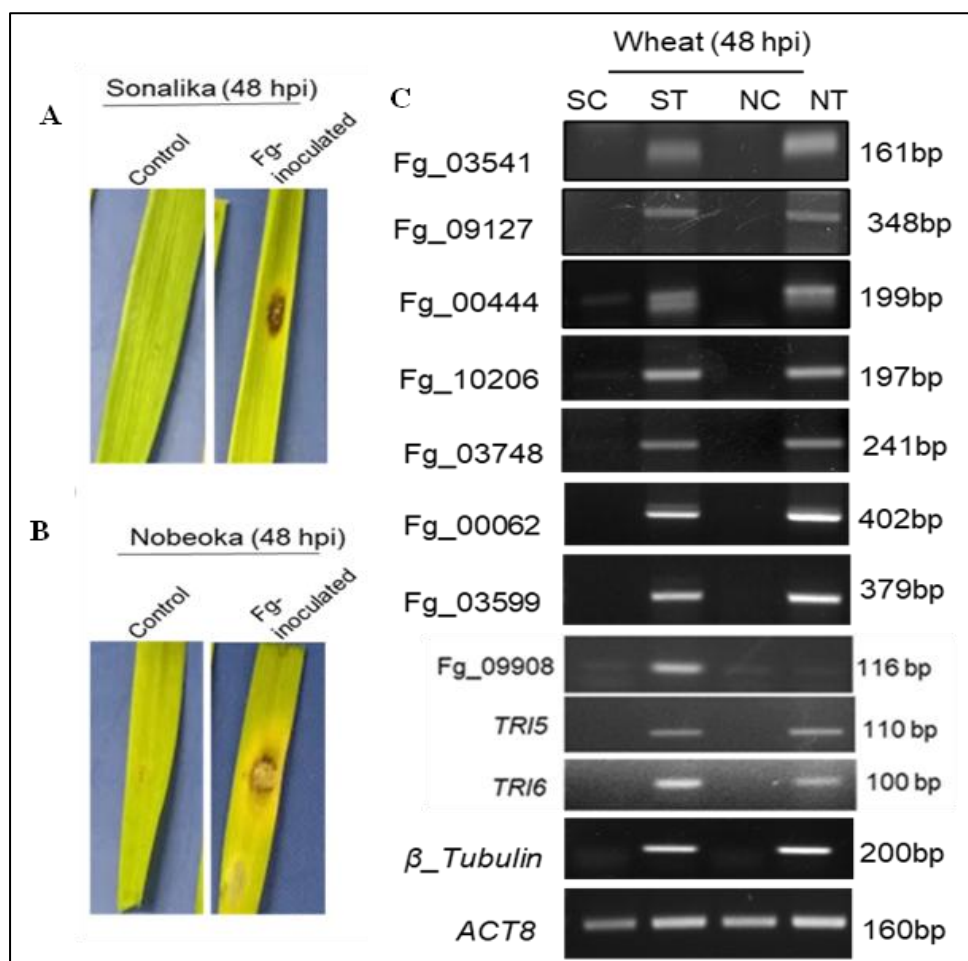


Figure 2.9 Disease phenotype of *F. graminearum* (Fg)-inoculated leaves of (A) Sonalika (susceptible) and (B) Nobeoka (resistant) wheat genotypes. (C) Candidate gene expression in *F. graminearum* (Fg)-inoculated Sonalika and Nobeoka compared to mock (water)-treated control. Plant-specific (*ACT8*) and fungal-specific (β -*Tubulin* and *GAPDH*) were used as internal controls.

2.3.6. Gene expression analysis of candidate effector genes during wheat head infection

The expression profile of the 16 effector and non-effector candidate genes was monitored by RT-PCR in the spikes of *F. graminearum*-inoculated Sonalika and Nobeoka genotypes at later time points of 48 and 72 hpi (**Figure 2.10**). All the 3 putative killer toxin genes— FGSG_00060, FGSG_00061, and FGSG_00062 belonging to the Kp4 superfamily were induced in wheat spikes in response to *F. graminearum*. The gene, FGSG_00060 expressed in wheat spikes alone but not in the leaves of Arabidopsis and wheat plants in response to *F. graminearum* suggesting a tissue-specific expression. Moreover, its expression was higher in the *F. graminearum* (Fg)-inoculated Nobeoka (FHB resistant) at 48 hpi and 72 hpi compared to *F. graminearum*-inoculated Sonalika indicating its possible role in virulence to interact with receptors like R- genes (Quarantin et al., 2019). Likewise, another candidate gene, FGSG_13281 did not express in both Arabidopsis and wheat leaves but showed higher mRNA transcript accumulation in wheat spikes at 48 and 72 hpi in response to the pathogen. Of the 16 gene candidates 2 genes— FGSG_11205 and FGSG_13281 expressed at 72 hpi alone (**Figure 2.11**) while the genes, FGSG_10603 and *TRI6* (used to check trichothecene expression) expressed at 48 hpi alone suggesting that these effectors might be temporally regulated. Whereas, FGSG_00061 and FGSG_03846 were induced in the spikes of the susceptible Sonalika genotype at 48 and 72 hpi suggesting their contribution to susceptibility.

Interestingly some of the gene candidates showed either reduced expression levels or negligible mRNA abundance at 72 hpi in the resistant wheat genotype, Nobeoka indicating them as putative virulence factors. The 4 gene candidates identified as virulence factors in this study are FGSG_00062, FGSG_07921, FGSG_04213, FGSG_10603, FGSG_03846, and *TRI6*. The remaining candidate genes showed upregulation in Sonalika suggesting their role as pathogenicity factors to promote pathogen growth in wheat.

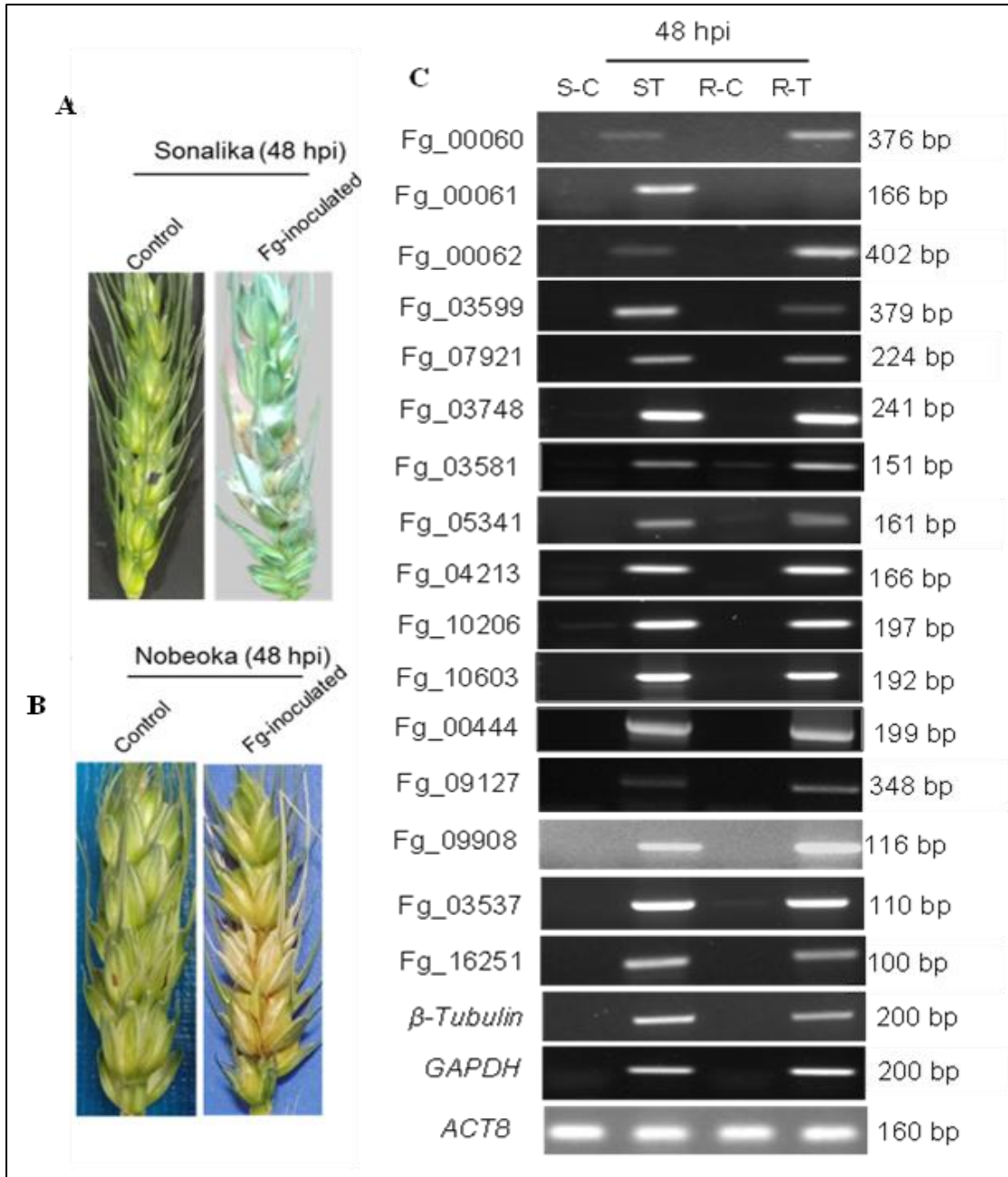


Figure 2.10 Reverse Transcriptase (RT-PCR) of effector and non-effector gene candidates in *F. graminearum* (Fg)-inoculated wheat spikes compared to mock (water)-treated control at 48 hpi. Disease phenotyping of *F. graminearum* (Fg)-inoculated spikes of (A) the susceptible, Sonalika (B) the resistant, Nobeoka and (C) candidate gene expression in *F. graminearum* (Fg)-inoculated spikes of Sonalika and Nobeoka at 48 hpi.

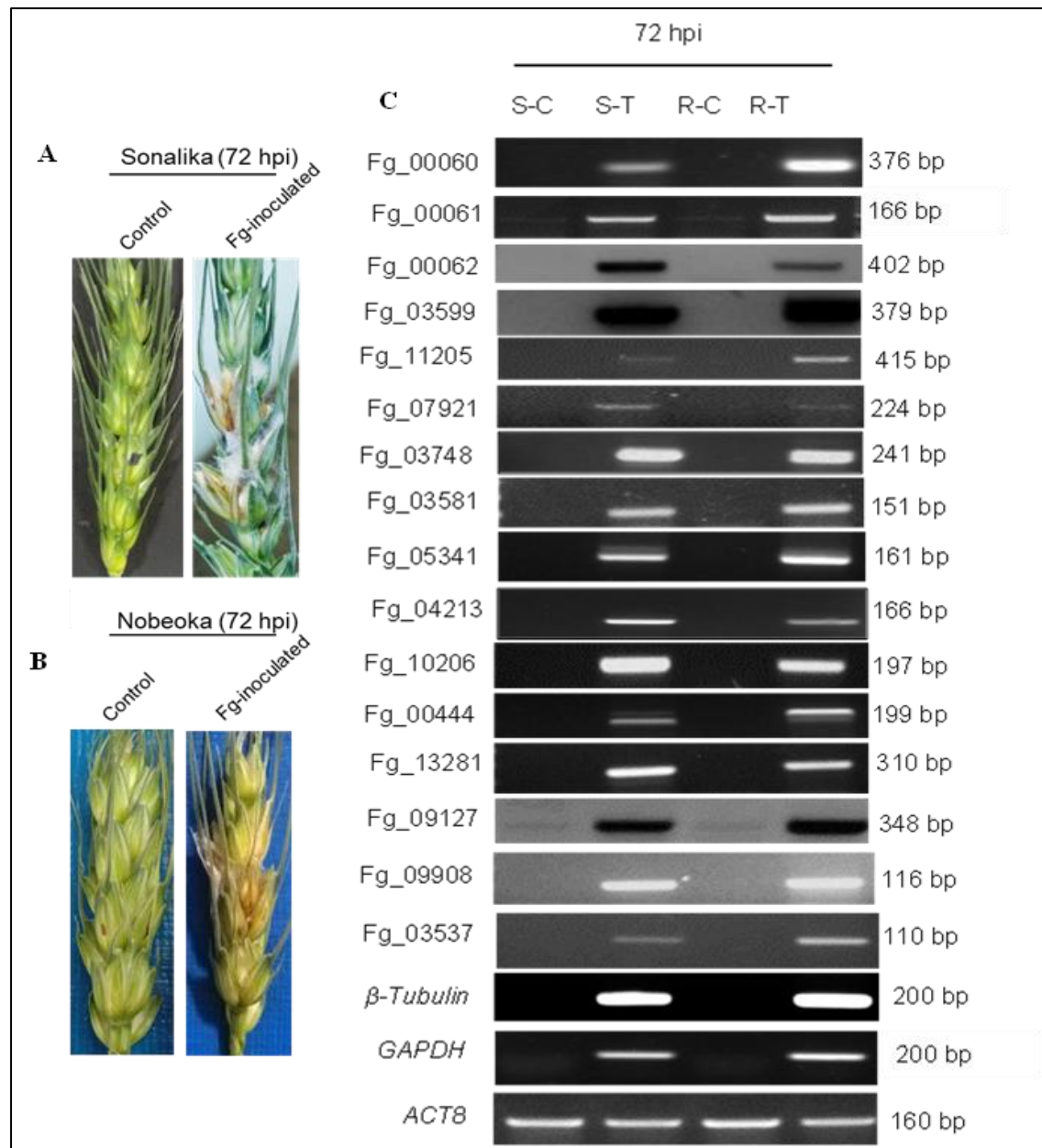


Figure 2.11 Reverse Transcriptase (RT) PCR of effector and non-effector gene candidates in *F. graminearum* (*Fg*)-inoculated wheat spikes compared to mock (water)-treated control at 72 hpi. Disease phenotyping of *F. graminearum*-inoculated spikes of (A) Sonalika (B) Nobeoka and (C) candidate gene expression in *F. graminearum* (*Fg*)-inoculated Sonalika and Nobeoka at 72 hpi. Plant-specific (*ACT8*) and fungal-specific (β -*Tubulin* and *GAPDH*) gene expression were used as internal controls. S-C: Susceptible Control; S-T: Susceptible-inoculated; R-C: Resistant Control; R-T: Resistant inoculated.

2.3.7. Real-time- quantitative (qRT-PCR) gene expression profiling of putative effectors in Arabidopsis and wheat heads

To analyze the expression of putative candidate genes at the early time point of infection (0, 6, 12, and 24 hpi), qRT-PCR were carried in the *F. graminearum*-inoculated leaves of Arabidopsis plants in comparison to the control plants mock-treated with water. The expression profile of a set of 5 putative effector genes— FGSG_03748, FGSG_00444, FGSG_09127, FGSG_09908, and FGSG_03599 were analyzed. The fold change gene expression levels ranged from ~1-fold change (6 hpi) to ~9-fold (24 hpi) for the 5 genes when compared to the controls.

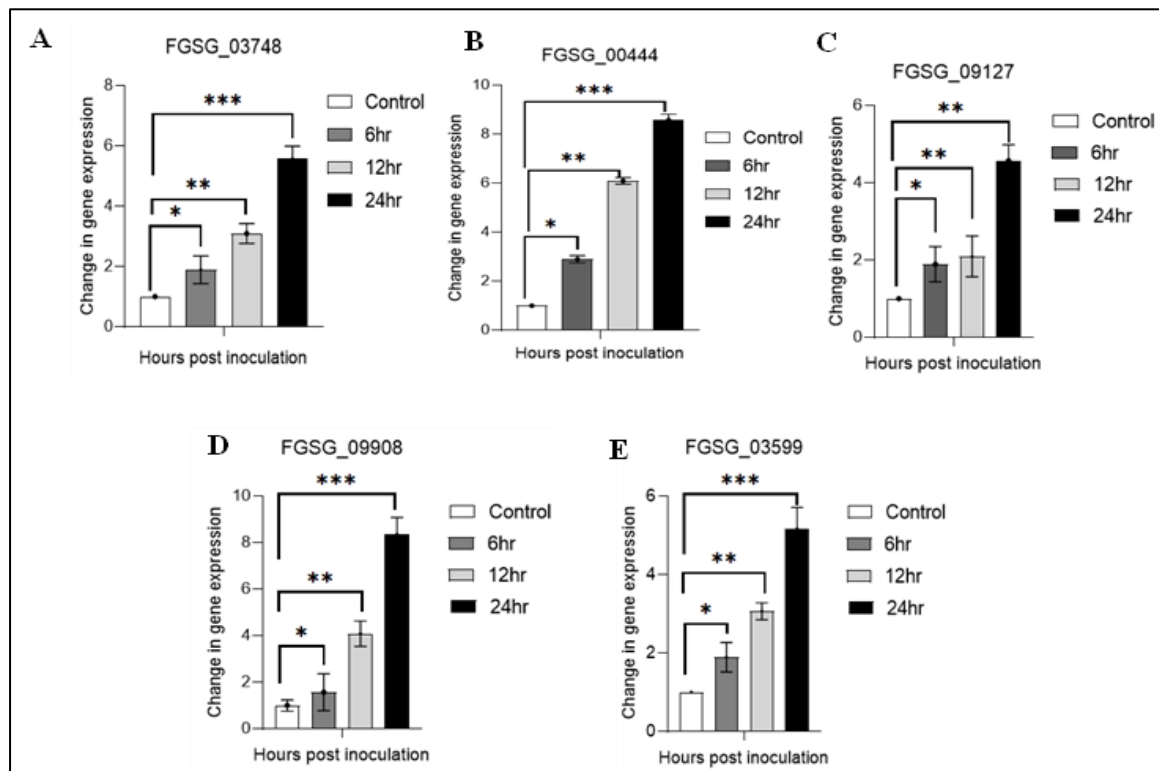


Figure 2.12 Real Time-quantitative PCR (qRT-PCR) validation of the effector genes in *F. graminearum* (Fg)-inoculated Arabidopsis (wild-type Col-0) leaves 0, 6, 12, and 24 hpi. The plant-Actin (*ACT8*) and fungal-specific β -Tubulin genes were monitored as internal controls for Rtq-PCR with no-template DNA control (NTC) as negative control. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. The level of significance of variation between samples is indicated as *P < 0.05; **P < 0.01; ***P < 0.001.

A significant increase in the fold-change was observed at early time points for all the genes from 6 hpi to 24 hpi except for the gene, FGSG_09127 which showed similar fold-change expression levels at 6 and 12 hpi. However, at 24 hpi, an increase with a 4.5-fold-change expression was observed. The fold change expression at 24 hpi was highest for all the genes with a log-fold change of 5, 9, 4.5, 9, and 5 respectively for the 5 genes– FGSG_03748, FGSG_00444, FGSG_09127, FGSG_09908 and FGSG_03599 compared to controls (**Figure 2.12**).

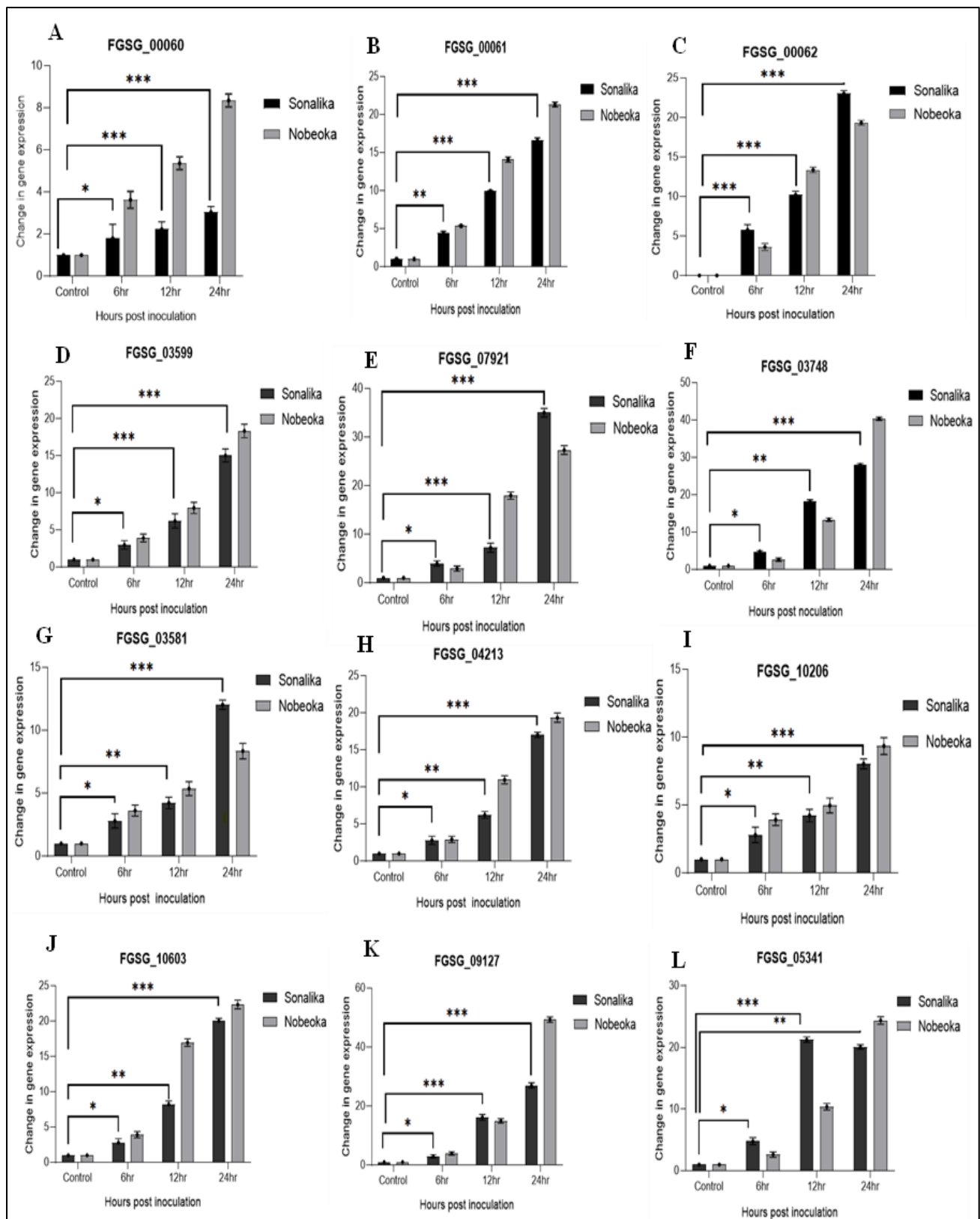
The expression of a set of 16 putative effector genes including the previously reported trichothecene genes– *TRI5* and *TRI6* was monitored at early time points of 6, 12, and 24 hpi by qRT-PCR in *F. graminearum*-inoculated Sonalika (FHB susceptible) and Nobeoka (FHB resistant) genotypes (**Figure 2.13**). A significant level of variation was detected between the samples at different time points of inoculation. Eleven out of the 16 putative genes showed higher expression levels at 6, 12, and 24 hpi in the resistant genotype, Nobeoka whereas 4 genes expressed at higher levels in Sonalika at 24 hpi compared to control. The mRNA transcripts of the putative effector genes– FGSG_00060, FGSG_00061, FGSG_03599, FGSG_03748, FGSG_05341, FGSG_04213, FGSG_10206, FGSG_10603, FGSG_00444, FGSG_09127, FGSG_09908, *TRI5*, and *TRI6* were up-regulated at all the time points suggesting that these genes could be putative pathogenicity factors. On the contrary, the mRNA abundance of FGSG_07921 and FGSG_00062 was reduced at 24 hpi in the resistant, Nobeoka genotype suggesting them as virulence factors with the possible interaction with the resistant genetic components in Nobeoka. The expression of the remaining putative effectors– FGSG_00062, FGSG_07921, and FGSG_03581 were higher at 24 hpi in Sonalika compared to the Nobeoka genotype suggesting their putative role in pathogenicity.

The expression of the genes FGSG_05341, FGSG_00062, FGSG_00444, FGSG_09908, *TRI5*, and *TRI6* were induced at 6 hpi with ~5-6-fold in Sonalika and gradually decreased at 12 hpi followed

by a higher fold change expression at 24 hpi. The RT-PCR results also showed higher expression at 48 and 72 hpi suggesting their strong role in the FHB infection. It was interesting to note that FGSG_10603 showed high expression levels at 6 hpi (~4-fold), 12 hpi (~17-fold), and 24 hpi (~22-fold). Similarly, the expression of FGSG_10206 showed a gradual increase initiating at 6 hpi (~4-fold), 12 hpi (~5-fold), and 24 hpi (~9-fold) in Nobeoka compared to the Sonalika. Likewise, FGSG_00444, a Bax-related gene showed a high-level gene expression starting at 6 hpi (~5-fold), 12 hpi (~10-fold), and then decreased at 24 hpi in Sonalika. Whereas, its expression in Nobeoka was low at 6 hpi, which increased significantly at 12 (~13-fold) and 24 hpi (~25-fold) as shown in **(Figure 2.13)**.

In our study, we found that the expression of all the (Kp4 superfamily genes)– FGSG_00060, FGSG_00061, and FGSG_00062 in wheat spikes but in a differential manner. At 6 hpi, the expression of FGSG_00060, FGSG_00061, and FGSG_00062 in Sonalika were found to be ~2-fold, ~4-fold, and ~5-fold respectively. At 12 hpi, the expression of these 3 genes in Sonalika increased twice the levels as observed at 6 hpi. At 24 hpi, the expression of FGSG_00061 and FGSG_00062 increased drastically by ~15-fold and ~22-fold respectively suggesting their crucial role in the pathogenesis of *F. graminearum*. Of all these 3 genes, FGSG_00061 peaked in its expression with ~21-fold at 24 hpi followed by FGSG_00062 and FGSG_00060 with ~18-fold and ~8-fold change expression respectively in Nobeoka as observed in **Figure 2. 13**.

A significant level of mRNA transcript accumulation of the genes– FGSG_09908 (~16-fold), FGSG_03581 (~13-fold), and FGSG_07921 (~35-fold) was observed in Sonalika at 24 hpi as shown in Fig 5, suggesting their role in the initial stages of pathogenesis. The expression of the gene, FGSG_03748 also elevated at all the time points in both the genotypes but with high expression of ~40-fold change at 24 hpi in Nobeoka. Another effector, FGSG_04213 showed ~3, ~11, and ~20-fold expression at 6, 12, and 24 hpi respectively in Nobeoka compared to Sonalika.



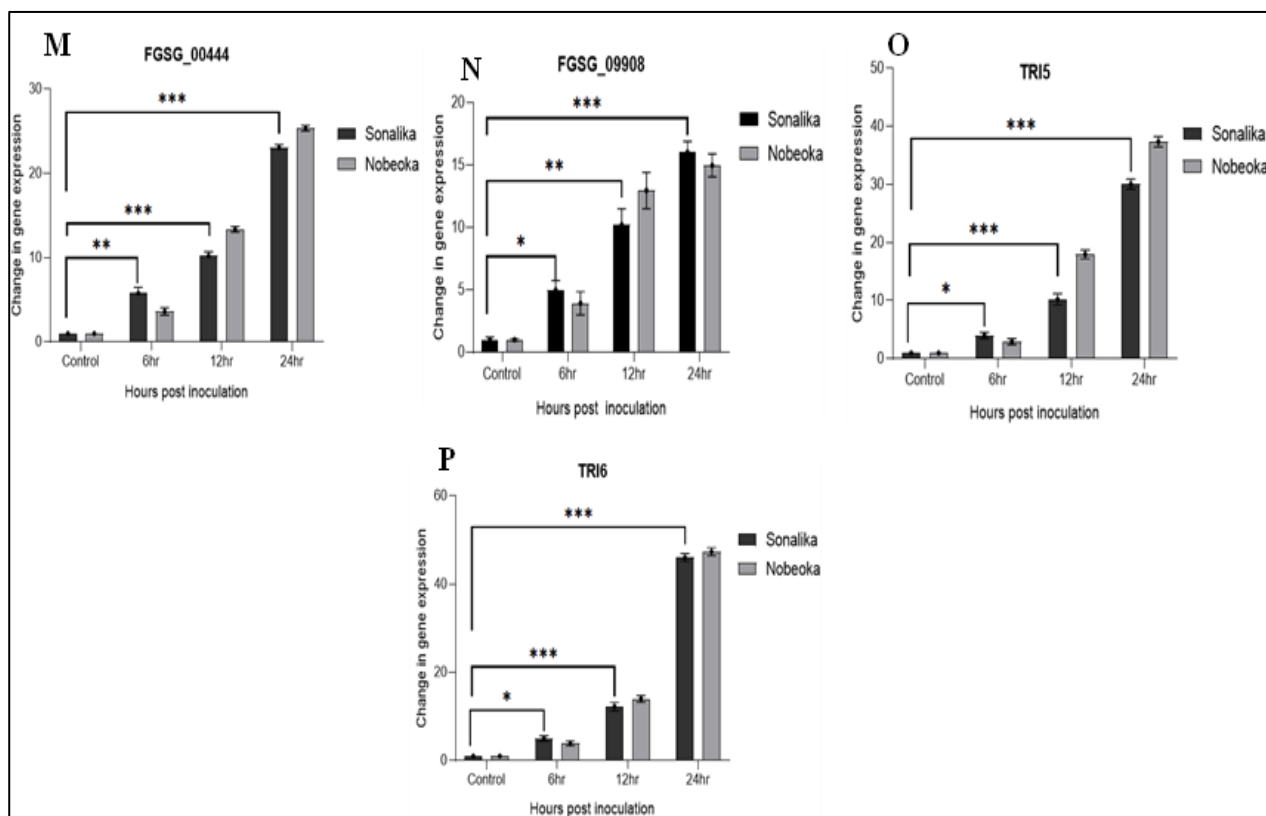


Figure 2.13 Validation of the putative effector genes by qRT-PCR in *F. graminearum* (Fg)-inoculated spikes of susceptible (Sonalika) and resistant (Nobeoka) genotypes at 0, 6, 12, and 24 hpi. The plant-Actin (*ACT8*) and fungal-specific β -Tubulin genes were monitored as internal controls for qRT-PCR with no-template DNA control (NTC) as negative control. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method. The level of significance of variation between samples is indicated as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Similarly, FGSG_03599 showed ~4-, ~8- and ~18-fold expression at 6, 12, and 24 hpi respectively in Nobeoka compared to Sonalika suggesting their crucial role in pathogenesis at all stages. Since *TRI* genes are mainly involved in DON production, the expression of *TRI5* and *TRI6* genes was evaluated in *F. graminearum*-inoculated plants of both resistant (Nobeoka) and susceptible (Sonalika) genotypes. The mRNA transcripts of *TRI5* and *TRI6* genes also increased significantly at all the time points with high fold-change values of ~40- and ~42 respectively at 24 hpi in Fg-inoculated Nobeoka suggesting their role in pathogenesis and virulence (**Figure 2.13 N-O**).

2.3.8. Microscopic and histological studies to assess fungal growth and H₂O₂ localization

Evaluation of the pathogen infection at the microscopic level was performed in a time-course study using trypan blue staining of Arabidopsis leaves inoculated with *F. graminearum*.

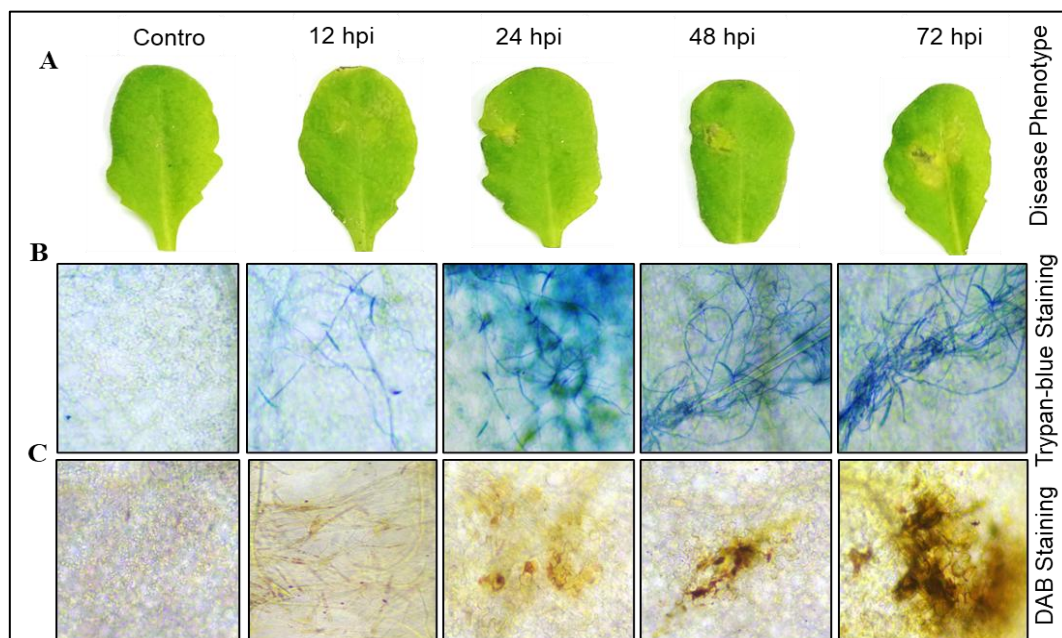


Figure 2.14 Microscopic studies to monitor *F. graminearum* (*Fg*) growth and host response in *Fg*-inoculated leaves of Arabidopsis (wild type Col-0) at 12, 24, 48, and 72 hpi compared to mock (water)-treated control. Representative images of (A) disease phenotype of *Fg*-inoculated Arabidopsis leaves infection; microscopic investigation of fungal growth in (B) trypan blue stained and (C) 3, 3'-diaminobenzidine (DAB)-stained *F. graminearum* (*Fg*)-inoculated Arabidopsis leaf tissues; Arrows indicate the *Fg* mycelium and spores. Magnification: 40x.

The fungal mycelial growth was compared between *F. graminearum* (*Fg*)-inoculated and mock (water)-treated Arabidopsis leaves at 0, 12, 24, 48, and 72 hpi. An increase in the mycelial growth and spread was observed at all the time points starting from 12 hpi in Arabidopsis leaves which correlated with the appearance of disease symptoms on the adaxial surface of the leaves as shown in (Figure 2.14 A) and (Figure 2.14 B).

The reddish-brown discoloration observed in the pathogen-challenged leaf tissue was shown as a defense mechanism resulting from the accumulation of ROS and antioxidant system responses

against *F. graminearum* infection in wheat (Khaledi et al., 2016). Similar discoloration of leaf tissue was observed starting at 12 hpi in response to *F. graminearum* in Arabidopsis with gradual increase at all the time points indicating the accumulation of ROS and/ H_2O_2 primed by the pathogen in Arabidopsis (**Figure 2.14 C**).

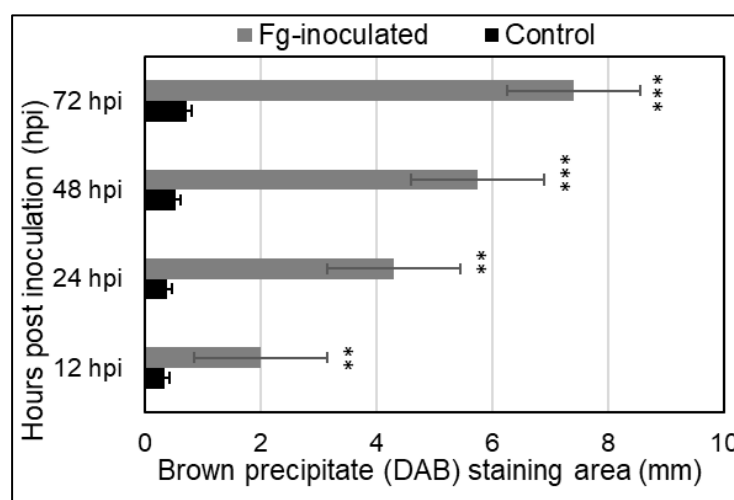


Figure 2.15 Assessment of H_2O_2 detected by DAB in control, *F. graminearum* (*Fg*)-inoculated *Arabidopsis* leaves, and quantification of the stained leaf areas. The graphs represent the mean and SD of three independent experiments.

Similarly, quantification of the brown precipitation of DAB staining in Arabidopsis leaves showed a significant progression due to ROS accumulation at all the time points in the *F. graminearum*-inoculated Arabidopsis leaves as shown in (**Figure 2.15**). These detached assays mimic the interactions that occur between fungus and wheat leaf tissues, hence offering distinct experimental circumstances. Wheat leaves were exposed to the *F. graminearum* inoculum, which might enter the leaves and spread infection. Their bioassay system identified differences in disease response between susceptible and resistant cultivars using damaged wheat leaves. Consequently, signs of disease growth on the infected plant include brown blotches as well as discolored, misshapen, necrotic, or chlorotic areas on leaves (Browne & Cooke., 2004; Browne et al., 2005). The *F. graminearum* infection on the leaves of the two wheat genotypes– Sonalika (susceptible) and

Nobeoka (resistant) at 0, 12, 24, 48, and 72 hpi also revealed rapid disease progression in Sonalika compared to Nobeoka (**Figure 2.16 A-B**) at 24 hpi and subsequent time point of inoculation. The microscopic examination for mycelial growth also showed similar progression with profuse mycelial growth in Sonalika from 24 hpi to 72 hpi compared to Nobeoka (**Figure 2. 17 A-B**). Though mycelial growth was observed in both the genotypes, a prominent level of symptom development was observed in susceptible wheat genotype, Sonalika.

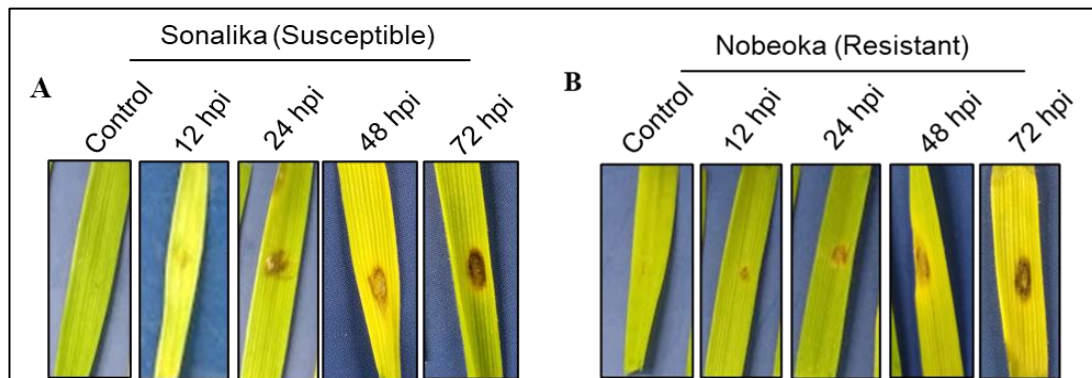


Figure 2.16 Detached leaf assay of *F. graminearum* (*Fg*)-inoculated leaves of the two wheat genotypes: Sonalika (susceptible) and Nobeoka (resistant) at 12, 24, 48, and 72 hpi compared to mock (water)-treated control. Representative images of disease phenotype of *F. graminearum* (*Fg*)-inoculated leaves of (A) susceptible, Sonalika and (B) resistant, Nobeoka.

The brown discoloration of the DAB-stained tissue resulting from potential ROS and/ H_2O_2 accumulation was monitored in both the wheat genotypes in response to *F. graminearum* inoculation, at different time points of infection conditions in comparison to the mock/control leaves. The brown discoloration of DAB-stained tissue, though observed in the leaves of both the genotypes, started as early as 12 hpi in the resistant genotype, Nobeoka compared to the susceptible genotype, Sonalika in response to *F. graminearum* (**Figure 2.17 C-D**).

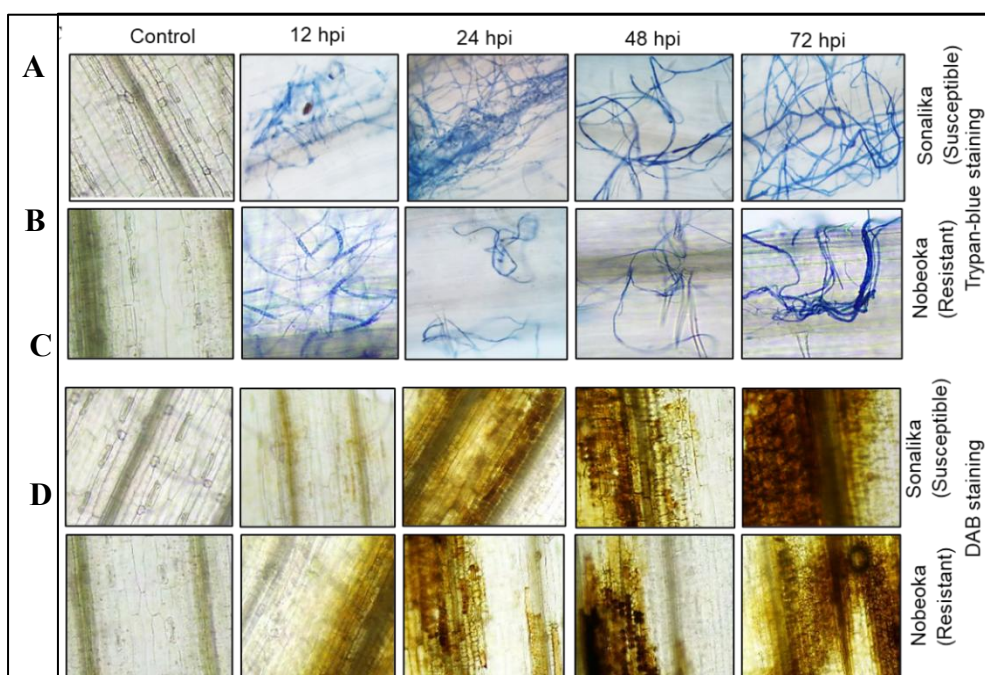


Figure 2.17 Histological studies of *F. graminearum* (*Fg*)-inoculated leaves of the two wheat genotypes: Sonalika (susceptible) and Nobeoka (resistant) at 12, 24, 48, and 72 hpi compared to mock (water)-treated control. Representative images of disease phenotype of *F. graminearum* (*Fg*)-inoculated leaves of (A) susceptible, Sonalika and (B) resistant, Nobeoka; (C) trypan blue stained and (D) DAB-stained *F. graminearum* (*Fg*)-inoculated tissues showing fungal growth.

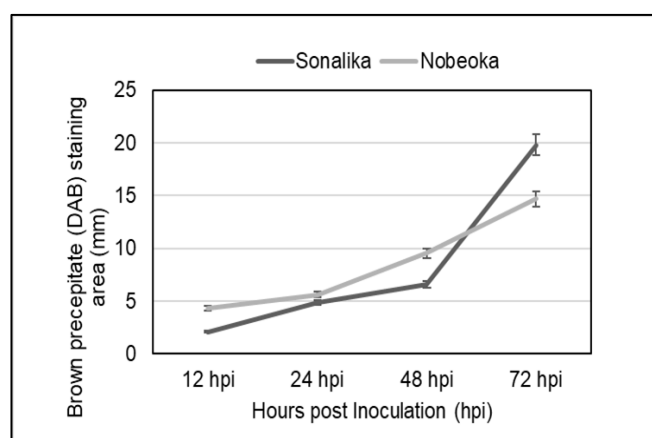


Figure 2.18 Visualization of H_2O_2 detected by DAB in control, *F. graminearum*-infected wheat leaves, and quantification of the stained leaf areas. The graphs represent the mean and standard deviation (SD) of three independent experiments.

Similarly, the quantification of the brown discoloration of DAB-stained tissue due to ROS accumulation also showed a similar result with relatively higher intensity at early time points ranging from 12 to 48 hpi in Nobeoka but with an increased level at 72 hpi in Sonalika in response to *F. graminearum* as shown in (Figure 2.18).

2.3.9. Monitoring Fusarium head blight (FHB) disease incidence on wheat spikes

FHB disease incidence was tracked on spikes of both resistant and susceptible wheat genotypes that were inoculated with *F. graminearum* at 7 and 10 dpi. Although disease incidence was noted on the *F. graminearum*-inoculated spikes of both wheat genotypes, the percentage of infected spikelets was significantly greater in the susceptible genotype, Sonalika, at all observed time points as shown in (Figure 2.19).

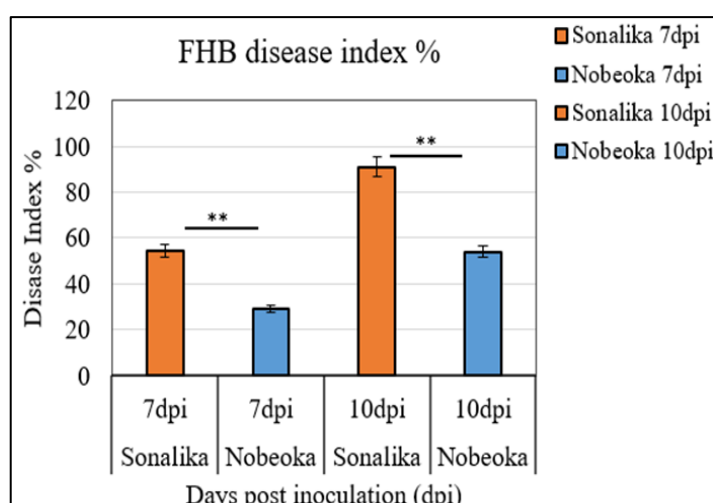


Figure 2.19 Comparison of FHB disease incidence on spikes of susceptible (Sonalika) and resistant (Nobeoka) wheat genotypes at 7 and 10 dpi. *F. graminearum* (*Fg*)-inoculated spikes of Sonalika showed a significant level of FHB disease compared to Nobeoka at 7 and 10 dpi. Statistical significance was tested by student-t test at different time points. The level of significance of variation between samples is indicated as **P < 0.01.

2.4 DISCUSSION

Fusarium graminearum being one of a phytopathogen capable of switching its mode of living from a biotrophic phase to a necrotrophic phase in its growth period (Brown et al., 2017). To establish effective infection and colonization in the host cells, the fungus is reported to secrete a repertoire of pathogenic molecules, often referred to as ‘effectorome’. Though previous studies involving transcriptome and proteome have predicted the effectorome of *F. graminearum* (Rampitsch et al., 2013; Lovelace et al., 2023), the information about the effectors with a role in pathogenicity and virulence is limited. Moreover, the predicting effectors based on specified effector attributes alone might eliminate the chances of detecting the non-effectors lacking those properties but carrying the effector function (Alouane et al., 2021). Therefore, a combination of techniques involving an *in-silico*-based gene search for putative effector and non-effector genes and gene expression in different host systems, accompanied by bioassays for virulence, would complement for effective detection of the pathogenesis and virulence genes of *F. graminearum*.

A genome-wide search in *F. graminearum* strain PH-1 led to the detection of several putative effector and non-effector genes, of which 16 genes were selected for gene expression studies based on the reproducibility at genomic and cDNA levels. These genes were tested through gene expression studies to confirm their role in pathogenicity and virulence. Of these 16 genes, 5 putative genes— FGSG_00060, FGSG_00061, FGSG_00062, FGSG-3599, and FGSG_11205 had been previously reported as putative effectors by (Lu & Edwards. 2016) and the two trichothecene genes (*TRI5* and *TRI6*) were used as reference genes to evaluate the expression of the potential genes found in this work. The gene expression studies carried out in the two host-pathogen interactions— one comprising *Arabidopsis* and *F. graminearum* pathosystem and the other comprising wheat and *F. graminearum* pathosystem in a time course study representing both early and late stages of infection led to the identification of putative pathogenicity and virulence factors. Of these, some gene candidates might be crucial for virulence while the remaining might be

employed by the pathogen at different stages of infection to ensure effective infection and establishment in the host. However, a few of the effectors were not expressed in *Arabidopsis*, though expressed in wheat suggesting that since the pathogen is highly adapted to the host, wheat, might be employing an arsenal of effectors to break down the effective barriers acquired by the host.

One of these genes has recently been characterized (Hao et al., 2020) and shown to be involved in the initial infection of wheat, though it was found to be not essential for the establishment of the disease. However, deletion of this candidate gene significantly reduced the initial infection and interfered with the type I resistance mechanism in wheat. Interestingly, FGSG_09908, a cAMP-regulatory protein (*CRP*) showed upregulation in the *F. graminearum* (*Fg*)-inoculated *Arabidopsis* as well as wheat leaves suggesting their role in the pathogenicity of the pathogen in accordance with the findings from a previous study. Likewise, the expression pattern of FGSG_09908 and *TRI6* genes were comparable at all the time points but with high-fold change expression in FGSG_09908 in both the wheat genotypes suggesting its role in virulence. This gene has a cAMP function that plays an important role during the pathogenesis of some fungal pathogens. But in *Fusarium* wheat spike infection this gene has been shown to reduce the virulence when present in mutant form (Paccanaro et al., 2017). Several of the selected effector candidates, including hypothetical proteins, exhibited conserved functional domains that have been related to pathogenesis in other fungal pathogens. Three of the candidate effector genes FGSG_00060, FGSG_00061, and FGSG_00062 carried a functional domain of *KP4* killer toxins. Killer toxins with a phytotoxic function and belong to a multigene family protein in *F. graminearum*. It's no. ranges from 1-4 genes/ *F. graminearum* genome (KP4L-1, KP4L-2, KP4L-3, and KP4L-4). *F. verticellioides* contain an additional 2 genes that are KP4L-5 and KP4L-6 (Vicente et al., 2022). While one of the effectors, FGSG_03599 had the characteristic CFEM (common in the fungal extracellular membrane) domain, the other candidate effector FGSG_00444 was found with

Apoptosis-linked gene 2 conserved domain cd16180. During plant-pathogen interactions, the CFEM proteins (found in numerous fungal extracellular membranes) have been shown to behave as cell-surface receptors, signal transducers, or adhesion molecules and they might be required at the initial stages of *F. graminearum*-infection in wheat irrespective of the genotype (Kulkarni et al., 2003; Chen et al., 2021). Similarly, Pth11 (a CFEM protein) in *Magnaporthe grisea* was previously shown to be involved in the colonization of the host by appressorial formation (DeZwaan et al., 1999). Considering the significant level and similar pattern of expression of the majority of the effector genes at different time points, the findings indicate that these effectors exhibit both spatial and temporal expression in the host to facilitate pathogen infection and spread in the host plants.

The sequences of effector proteins are extremely diverse, and the majority of them do not exhibit any sequence or domain resemblance to recognized proteins. However, only a few recent studies on effector proteins have demonstrated that these conserved 3-dimensional protein structures may provide evidence of similar functions for the proteins with the common protein folds. The protein modelling structures were obtained based on template-free modeling by TrRosetta. Enriched folds have been discovered using genome-wide structural modelling of putatively secreted proteins in plant-pathogen interactions, which will be used to anticipate effectors and their respective functions (Martínez-Cruz et al., 2021). These folds could be involved in chaperone association, protein secretion, or on a broader scale, virulence functions. It is crucial to note that there are only a handful of currently known structural effector families (Andreeva et al., 2020), and many more folds/motifs need to be recognized, as demonstrated by the comprehensive study of protein folds found in nature.

Some of the potential effector and non-protein structures were identified based on TrRosetta match with the previously identified effector protein structures from other fungal pathogens, specifically with *M. oryzae*, *U. maydis*, and *S. cerevisiae*, and the Protein Data Bank (PDB). Homologous

matches were identified by searching the *F. graminearum* effector sequences against the Protein Data Bank database with BLASTP. By using molecular docking to anticipate effector protein interactions with plant receptors, projected effector protein structures will be useful in improving our comprehension of effector–plant interactions (Rozano et al., 2024).

During infection, the accumulation of H₂O₂ in plant tissues in response to pathogen infection is demonstrated as a defense mechanism to trigger cell death-inducing signals to confer resistance responses in the majority of the plant-pathogen interactions (Kuźniak et al., 2014). In our study, the DAB-stained leaf samples of both *Arabidopsis* and wheat demonstrated reddish-brown tissues with a possible accumulation of H₂O₂ in the *F. graminearum*-inoculated leaf samples coincide with the findings of (Khaledi et al., 2016). Early ROS build-up in the resistant genotype, Nobeoka indicated rapid activation of defense response in Nobeoka. The increased H₂O₂ levels in the Nobeoka-*F. graminearum* interaction may be attributable to significant cell death to limit pathogen development in the latter stages of infection. For example, the protein called *Pep1*, which is vital for penetration and is derived from the biotrophic pathogen *U. maydis*, suppresses plant immunity by inhibiting the activity of a maize peroxidase known as *POX12*. This inhibition is critical for the production of ROS in the plant (Friesen et al., 2008; Doehlemann et al., 2009).

In contrast, necrotrophic effectors, which are also referred to as host-selective toxins, cause host susceptibility by inducing cell death and promoting disease (Vleeshouwers & Oliver, 2015). *Stagonospora nodorum* and *Pyrenophora tritici-repentis*, two necrotrophic wheat pathogens, produce an effector called ToxA that triggers severe necrosis in wheat plants with dominant sensitivity genes (Lu et al., 2014). The observation of elevated H₂O₂ levels in the leaf tissues of *F. graminearum*-inoculated Sonalika at 72 hpi compared to *F. graminearum*-inoculated Nobeoka in this study could be resulting from the necrotrophic phase of *F. graminearum* at the later stages of infection. Further, bioassays carried out to monitor the FHB incidence in the two wheat genotypes, confirmed the rapid progression of the disease in the susceptible genotype, Sonalika compared to

the resistant genotype, Nobeoka in response to the fungal pathogen. Apart from its potential use as a quick pre-screening method in breeding, the detached leaf test has the benefit of precise measurement of the particular resistance. This might also make it possible to clarify the genetic basis of FHB resistance (Brown & Cook, 2004). The detached leaf test stimulates the interaction between the *F. graminearum* and the wheat leaf, hence offering distinct experimental circumstances. According to Browne & Cooke (2005), a resistant response rather than susceptibility may be indicated by necrotic lesion(s) following initial infection in barley (*Hordeum vulgare* L.) and subsequent stoppage of pathogen development in the detached leaf assay. (Kumar et al., 2011).

Chapter. 3

RNAi-mediated silencing of the candidate effector gene for virulence function in *F. graminearum*

3.1 INTRODUCTION

RNA interference (RNAi) has been used as a potential approach to combat pest and pathogen attack to ensure global food security (Machado et al., 2018; Sarkar & Roy-Barman, 2021). The small-interference RNA (siRNA)-based gene silencing serves as a reverse genetics tool in analyzing the function of a gene and is being extensively utilized in functional genomic studies in plants and other eukaryotic organisms (McGinnis, 2010). Though various crop protection strategies are being adopted along with the application of demethylation inhibitor (DMI) fungicides to reduce yield losses and improve resistance to FHB, there is no single method that offers absolute resistance to the pathogen. With the advent of modern techniques such as RNA interference (RNAi), it is now possible to dissect the underlying mechanism of the pathogenicity of *F. graminearum*.

The siRNA technique involves silencing target genes using double-stranded RNA (dsRNA) to achieve highly specific post-transcriptional regulation of target genes in an organism (Dalakouras et al., 2024). The specific gene silencing is reported to be induced by either mRNA degradation or suppression through the application of precursor long, non-coding double-stranded RNAs (dsRNA) that share sequence complementarity with the mRNAs of the target genes. Since the technique involves spraying of the siRNAs, it is also regarded as spray-induced gene silencing (SIGS). SIGS represents a contemporary, potential, non-transformative, and environmentally sustainable pest and pathogen management strategy wherein, naked or nanomaterial-bound dsRNA is applied to leaves (Koch et al., 2016) and young fruits (Wang et al., 2016) to induce selective knockdown of pathogenicity genes.

The cyclic AMP (cAMP)-dependent signaling pathway is crucial for signal transduction of various differentiation related as well as infection processes in numerous phytopathogenic fungi. The cAMP/ protein kinase A (PKA) signaling in *F. graminearum* and *M. oryzae* is essential for surface sensing, appressorium morphogenesis, turgor generation, and regulation of plant infection (Li et

al., 2012; Yan & Talbot, 2016). A crucial signaling pathway essential for the virulence of plant fungal pathogens is the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathway (Turrà et al., 2014).

In fungal phytopathogens, this pathway governs significant morphological transformations and the enhancement of virulence factors in response to the host environment. This regulation is vital for managing developmental transitions during infection, such as recognizing and penetrating the host surface, invasive growth, and forming reproductive structures. Furthermore, the pathway is crucial for nutrient sensing in yeast, influencing carbohydrate metabolism, the cell cycle, growth, and development (Zaman et al., 2008). Additionally, the cAMP-PKA pathway is known to control a wide range of fungal processes, including morphological changes, sexual reproduction, secondary metabolism, and responses to various nutritional signals (Hu et al., 2014; Caza & Kronstad, 2019). The activation of the catalytic subunit of PKA occurs only when cAMP binds to the regulatory subunit of the PKA pathway. In this activated form, the PKA can initiate numerous downstream factors (Li et al., 2007). In phytopathogenic fungi like *M. oryzae* and *Ustilago maydis*, cAMP signaling is a vital trigger for pathogenesis (Xu et al., 1997). cAMP-PKA signaling is essential for forming host penetration structures in many plant-pathogenic fungi, including *F. graminearum* (Bormann et al., 2014). Evidence also suggests that this pathway is involved in post-penetration invasive growth, as strains lacking cAMP-PKA components cannot induce disease after being inoculated into experimentally damaged leaf tissue (Li et al., 2017). In *F. graminearum*, studies have shown that the only intracellular target of cAMP is the regulatory subunit of PKA with a potential role in fungal growth regulation, sexual reproduction, sporulation, and mycotoxin production. It was also demonstrated that the deletion of the regulatory subunit of *F. graminearum* was not lethal but the mutant form of this gene resulted in defective sporulation and fungal growth along with reduced virulence (Li et al., 2018). Our previous studies also revealed an up-regulation of the putative *CRP* (FGSG_09908) gene transcripts in *F. graminearum*-challenged wheat and

Arabidopsis (Fatima et al., 2024). Therefore, we hypothesized that *CRP* might be involved in the pathogenicity of *F. graminearum*. Gene silencing studies through siRNA-mediated knockdown of the putative *CRP* (FGSG_09908) gene was performed by SIGS technique in *F. graminearum*. Further, to confirm the findings, *CRP* gene knockdown was also carried out in a different host-pathosystem comprising rice and *M. oryzae*, and the findings are presented.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and growth conditions

The plant material comprising wheat, rice, and *Arabidopsis* were obtained from the National Bureau of Plant Genetic Resources (NBPGR), New Delhi; Indian Rice Research Institute (IRRI), Hyderabad, and the Department of Plant Sciences, UoH, India, respectively. The seeds of Sonalika, a susceptible wheat genotype to FHB disease (Kumar et al., 2021), the model plant, *Arabidopsis* (*Arabidopsis thaliana* ecotype (Col-0), and BPT-5204 (Samba Mahsuri), a susceptible indica rice (*Oryza sativa* L.) to blast disease (Naik et al., 2021) were sterilized as described previously (Fatima et al., 2024) with the exception of the duration of bleaching treatment, which was 10 min in wheat and 5 min in rice. The *Arabidopsis* plants were grown for 4–5 weeks in growth chambers at 20 ± 4 °C Day /night, 16/8 h light/dark cycle, with 75% relative humidity. Whereas rice and wheat plants were grown with a photoperiod of 12:12 light/dark photoperiod, with the exception of the temperature regime of 26 °C in wheat and 28 °C in rice plants.

3.2.2 Fungal inoculum preparation

The *F. graminearum* strain TN01 was isolated and maintained as described earlier (Fatima et al., 2024). To prepare liquid cultures for sporulation, 4 g of mung bean seeds were washed and boiled for 5 min in 100 mL autoclaved distilled water, and cultures were kept with constant shaking at 150 rpm at 26 °C for 5 days. The macroconidia were passed through a 4-layered sterile cheesecloth as described previously (Makandar et al., 2006). The autoclaved double-distilled water was used for both washing and resuspension of the fungal conidia. From the Indian Rice Research Institute (IRRI), Hyderabad, the *M. oryzae* strain was procured. The oatmeal agar (OMA) purchased from HIMEDIA® was utilised for the subculturing and sporulation of the fungus on petri plates in the dark for 8–10 days at 26 ± 2 °C. Ten days subsequent to inoculation, the fungal mycelia were scraped and homogenized by vortexing. The homogenized mixture was filtered using sterile cheesecloth to

collect the conidia. The filtrate was centrifuged for 7 min at 7000 rpm. The spores were suspended in 10 mL autoclaved distilled water in a 15 mL sterile Falcon tube. Spore quantification was performed by using a hemocytometer (Neubauer, Marienfeld, Germany) under a light microscope. The conidial concentration was adjusted to a working concentration of 10^5 /mL for the experiments performed in this study.

3.2.3 Selection of the target gene and phylogenetic analysis

The nucleotide sequence of FGSG_09908 (GenBank accession no: XM_011320512), representing the cAMP-dependent protein kinase regulatory subunit of the PKA signal pathway, was retrieved by BLAST search from the NCBI database (<https://www.ncbi.nlm.nih.gov/>). Homologous sequences of the cAMP regulatory subunit of PKA from other ascomycete fungal pathogens like *F. verticillioides*, *Fusarium poae*, *M. oryzae*, *Colletotrichum truncatum*, and *Neurospora crassa* were retrieved from NCBI to check the sequence similarity for phylogenetic analysis. Phylogenetic relatedness was assessed among these fungi for their regulatory subunit of cAMP-dependent protein kinase using the maximum likelihood method by using MEGA 11 (Tamura et al., 2004) with a percentage of replicate trees of the bootstrap test (1000 replicates).

3.2.4 Designing siRNA primers and generation of siRNA duplex

An artificial siRNA that targets the mRNA sequence of the cAMP-regulatory subunit *CRP* gene of *F. graminearum* was designed by using the online available RNAi designer software (<https://sg.idtdna.com/>) to design ready-to-use gene-specific siRNA. The same was purchased from IDTA DNA Technologies. The primer sequences of the sense and antisense strands of the *CRP* gene are: 5'-rGrArCrCrArArCrCrCrGrUrGrArArGrCrUrCrUrArUrArAG-3' and 5'-rUrCrUrUrArUrArGrArGrCrUrUrCrArCrGrGrGrUrUrGrGrUrCrArA-3'.

The primer sequences of the sense and antisense strands of scrambled siRNA sequence used as control are: 5'-*rGrArCrCrArGrGrArTrArArArTrGrTrCrTrCrCrCrGrTrArUrCrAA*-3' and 5'-*rGrArCrArGrUrUrCrTrGrGrArGrTrCrGrArCrGrUrUrArCrUrArTrT*-3'. The primer-Blast tool (<https://www.blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to design gene-specific oligonucleotides from the coding sequence (CDS) of *F. graminearum* PH-1 genome for Reverse Transcriptase (RT), and real-time quantitative PCR (qRT-PCR). The primer sequences, along with their predicted amplicon sizes and annealing temperatures, are presented. A siRNA control (scrambled or unrelated siRNA) with zero sequence similarity to the *F. graminearum* genome in any of the sequences in the database was also purchased from the same authorized company. To generate the siRNA duplex with symmetric 3' overhangs, the sense and anti-sense strands were annealed in an annealing buffer (50 mM Tris, pH 7.5-8.0, 100 mM NaCl) with DEPC water, following the instructions provided with the primer (Khatri & Rajam, 2007).

3.2.5 Fungal mycelial growth and biomass (fresh and dry weight)

The germinating conidia of *F. graminearum* and *M. oryzae* treated with different concentrations of siRNA were assessed for mycelial growth and inhibition, induced by siRNA treatment as previously described (Khatri & Rajam, 2007). In the *F. graminearum* growth assay, 500 µL of 10⁵ conidia /mL were treated in 10 mL of liquid PDB medium (HIMEDIA®) with and without *CRP* siRNA treatment. Whereas in the *M. oryzae* growth assay, 300 µL of 10⁵ conidia /mL were treated in 5 mL of liquid PDB medium (HIMEDIA®) with and without *CRP* siRNA treatment. A 3 µL of 10⁵ germinating conidia/mL of untreated control and 10, 15, 25, 35, 50, 75, and 100 nM siRNA-treated samples were inoculated in the center of Petri plates containing PDA medium. The 48 hpt up to 7 days was chosen for measuring the diameter of mycelia. For each colony, three diameters were measured at right angles to one another, and the mean diameter was obtained for that colony. The colony diameter experiment was repeated three times.

The inhibition mycelial growth was measured as the percent decrease in diameters of the siRNA-treated colony compared with the control colony diameters. The percentage reduction was calculated as follows:

$$\text{Percentage reduction} = 100 - \frac{\text{Diameter of the colony formed from siRNA-treated conidia}}{\text{Diameter of the colony formed from the control conidia}} \times 100$$

The fresh and dry weights of mycelial biomass were assessed in both siRNA-treated and control samples of *F. graminearum* and *M. oryzae* at 48 hpt. For the same, 500 µL of 10⁵ conidia /mL were treated in 10 mL of liquid PDB medium (HIMEDIA®) with and without *CRP* siRNA, and for the estimation of the Fungal biomass, samples were incubated in a rotary shaker at around 150 rpm at 28 °C. This was done by filtering the mycelium from the liquid medium using a double layer of cheesecloth and placing it on pre-weighed Whatman filter paper. At 100 °C for 24 h, the mycelium was then dried in an oven before being weighed again. The dry biomass was determined by deducting the average weight of the Whatman filter paper (calculated from 6 strips dried at 100 °C for 24 hpt and weighed) from the weight of the filter paper with mycelia. Each experiment for the siRNA-treated and control samples was conducted twice in triplicate.

3.2.6 *In-vitro* treatment of *F. graminearum* spores with siRNA in liquid medium

The *in-vitro* treatment involved the co-incubation of *F. graminearum* TN01 along with the purified siRNA in both solid and liquid media. In 2 mL of PDB medium (HIMEDIA®), 100 µL of *F. graminearum* conidia suspension (10⁵ conidia/mL) were grown in the liquid medium. Various amounts of siRNA were diluted in sterile 1x TE. The *CRP*-siRNA was added to the fungal conidia in the liquid media for uptake of siRNA by the fungal conidia.

3.2.7 Conidial germination counts and aerial hyphal growth

Fungal conidia were germinated by incubating the cultures with a concentration of 10^5 /mL for 6 h at 26 °C at 200 rpm. The *CRP* siRNAs were added to the liquid culture medium to obtain a final concentration of 10, 15, 25, 35, 50, 75, and 100 nM and the cultures were incubated for 6 h. The germinating conidia were then fixed using 5% glutaraldehyde to record percent germination and the germ tube growth measured as length (mm). These experiments were repeated thrice with three replicates in each experiment with appropriate controls comprising untreated spore cultures and spore cultures supplemented with 35 nM unrelated siRNA. These cultures were maintained at 26 °C for 7 days for measuring the mycelial growth as diameter (cm).

3.2. 8 *CRP*-siRNA application, fungal inoculation, and pathogenicity assay

At the anthesis stage, heads or spikes of Sonalika plants were spray-treated with the *CRP*-specific siRNA by using sterile spray bottles. At 24 hpt, the siRNA-treated spikes were point-inoculated with *F. graminearum* conidial suspension as described previously (Makandar et al., 2012). Central spikelets on each side of the rachis were inoculated with 10 µL of 10^5 /mL fungal conidia per spikelet. Three spikes per plant were inoculated. Mock-treated spikes with water served as controls. The spikes were covered with a zip-lock for 2 days. For detached leaf assay, leaves from four-week-old wheat plants were sprayed with siRNA. At 24 hpt, *F. graminearum* spores were drop-inoculated with 10 µL of conidial suspension of concentration 10^5 conidia/mL on the adaxial surface of wheat leaves following the siRNA treatment (Brown et al., 2005; Gaffar et al., 2019).

Likewise, a detached leaf assay was carried out in *Arabidopsis* on four-week-old leaves of *A. thaliana* Col-0 as described previously (Gaffar et al., 2019). Leaves were sprayed by using the spray flask. Each leaf was sprayed evenly with 3-4 puffs with 100 nM siRNA and incubated at room temperature for 24 h. Subsequently, the siRNA-treated leaves were drop-inoculated with 20 µL of *F. graminearum* conidial suspension of concentration 10^5 conidia/mL.

In rice, for initial optimization, the detached leaves were sprayed locally with 50, 75, and 100 nM of siRNA at 24 h prior to *M. oryzae* inoculation. Later, 100 nM of siRNA was sprayed in local and distal regions of abraded leaves to check for systemic nature, as described previously (Koch et al., 2016). Rice leaves were subsequently inoculated with *M. oryzae* spore suspension at a concentration of 10^5 conidia/mL containing 0.05% Tween-20 (Park et al., 2012). The disease phenotype on the leaves was recorded at 5 and 7 dpi.

The controls comprised mock (autoclaved distilled water)-treated followed by *F. graminearum*-challenged leaf and spike samples. The treated samples were placed in Petri plates containing moistened filter paper for 5 days at room temperature and the plates were sealed with parafilm. The experiments were carried out in three biological replicates. At 5 dpi, the necrotic lesion size (mm) was measured in all the samples. Images were taken and the observed lesion area was calculated as the product of the length and width of the lesions using ImageJ software (Schneider et al., 2012).

3.2.9 Total RNA extraction, cDNA synthesis and RT-PCR

Total RNA was extracted from the treated and control fungal and plant samples at different time points by using RNAiso from (TAKARA) following the manufacturer's instructions. The cDNA from 1 µg of RNA was prepared using a cDNA synthesis kit (TAKARA). RT-PCR was performed to compare mRNA transcript levels of the *CRP* gene at different time points in untreated control and siRNA-treated samples. The thermal conditions for RT-PCR were followed as: initial denature at 95 °C for 30 s, followed by 30 cycles of final denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and concluded with an initial extension at 72 °C for 30 s and final extension at 72 °C for 5 min. Fungal β -*Tubulin* and β -*Actin* were used as the internal control.

3.2.10 qRT-PCRs for transcript abundance following siRNA treatment

The cDNA was diluted 20 times with distilled water and utilized as a template for RT-qPCR, which was performed in a 96-well plate using the Syber green real-time PCR detection system (Applied Biosystems). Primers used in the study are listed in (Table 3. 1). The reaction included 5 µL of SYBR green SuperMix, 1 µL of cDNA template from each sample, 0.5 µL of 10 µM primer, and 3 µL of nuclease-free water. The thermal cycle consisted of three stages: initiated by one cycle of 95 °C for 20 s, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s (PCR stage), and concluded with a final melt curve stage: 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. *β-Tubulin* (Fudal et al., 2007) and *β-Actin* (Wilson et al., 2010) genes were used as internal controls. The experiment was repeated three times. Relative fungal target gene transcription was calculated using the $2^{-\Delta\Delta CT}$ method. (Livak & Schmittgen, 2001).

Table 3.1. The list of primer sequences along with their expected amplicon size and annealing temperatures.

S. No	Name	Primer Sequence	Amp size (bp)	Annealing Temp (°C)
1	<i>CRP</i>	F: ACTTTCCGYCGCATCYTSAT R: CAATCTTKGATCGCTCGTATG	118 bp	55 °C
2	<i>β-Tubulin</i>	F: CTGCCATCTTCCGTGGAAAGG R: GACGAAGTACGACGAGTTCTTG	200 bp	53 °C
3	<i>β-Actin</i>	F: ACAATGGTTTCGGGTATGTGC R: CGACAATGGACGGGGAAGAC	200 bp	53 °C

3. 2.11 Statistical analysis

Statistical analysis was conducted using "GraphPad-Prism" software. The one-way analysis of variance (ANOVA) test was used to determine whether the differences in the average responses of the treatments were significant. P-values less than 0.05 were considered as significant. The student's t-test was used to assess the significance of individual treatments carried out in triplicates.

3.3. RESULTS

3.3.1 Phylogenetic analysis of *CRP* sequence

The homologous sequences of the cAMP regulatory subunit of PKA from other ascomycete fungal pathogens such as *F. verticillioides*, *F. poae*, *M. oryzae*, *C. truncatum*, and *N. crassa* were checked for sequence similarity using the maximum likelihood method. The phylogenetic analysis revealed high conservation of the cAMP regulatory subunits across all these ascomycete fungal members for the *CRP* gene shown in (Figure 3.1).

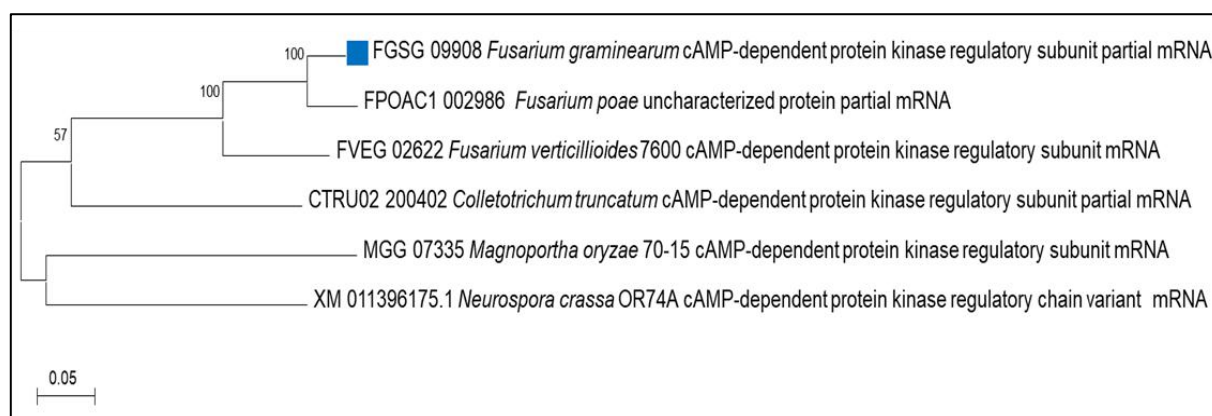


Figure 3. 1 Phylogenetic analysis of *CRP* (cAMP-dependent protein kinases regulatory subunit protein) sequences from multiple fungal species.

3.3.2 *F. graminearum* colony growth and spore morphology post-siRNA treatment

In order to assess the effect of siRNA treatment on the fungal growth and morphology, the *in-vitro* studies were performed with siRNA-treated *F. graminearum* conidial cultures supplemented with different concentrations of siRNA (0, 10, 15, 25, 35, 50, 75 and 100 nM) in the medium. In general, the mycelia spread from siRNA-treated spores was reduced compared to untreated control. The effect of siRNA-induced growth inhibition increased as the siRNA concentration increased. A 30% reduction in mycelial growth was observed in 25 nM siRNA-treated samples as compared to 35 nM with a 50% reduction in colony diameter at 5 dpt. In 75 nM treated samples, the diameter of the colonies reduced significantly compared to that observed at 100 nM. Of all

the concentrations, 35 nM concentration showed maximum inhibition at 5 dpt. Further at 7 dpt, colony growth in diameter and morphology remained unaltered even after increasing the concentration of siRNA to 100 nM, similar to the untreated control sample as shown in (**Figure 3.2**). In addition to this, the reduction in aerial hyphal growth on the plates was observed in siRNA-treated PDA plates. A significant reduction in growth measured as diameter was observed as compared to the untreated samples.

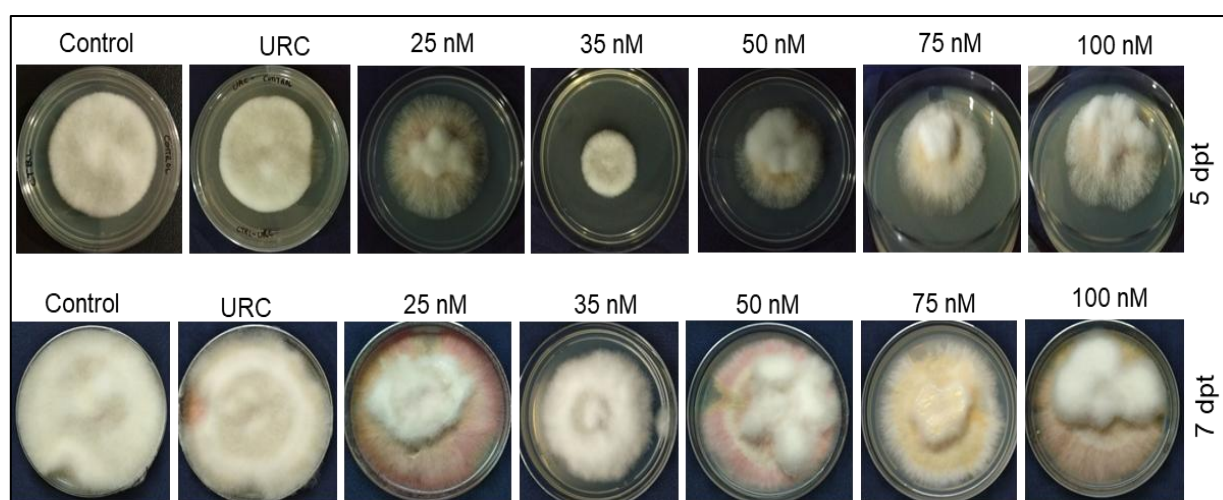


Figure 3. 2 The effect of siRNA on fungal growth and morphology of *F. graminearum* on PDA medium.

The results of mycelial fresh and dry weight further revealed that mycelial growth was significantly reduced by the *CRP*-specific siRNA-treated samples compared to control samples. Though the biomass reduced in all siRNA-treated samples, a prominent reduction was observed in case of mycelial wet-weight compared to its dry-weight. The reduction in total biomass in siRNA-treated samples was maximum at 35 nM in the case of wet weight. A similar pattern of biomass reduction was also recorded from 25 nM to 75 nM in dry-weight samples. Overall, the exogenous application of siRNA resulted in reduced growth of mycelia of *F. graminearum* on PDA culture plates as graphically shown in (**Figure 3.3**).

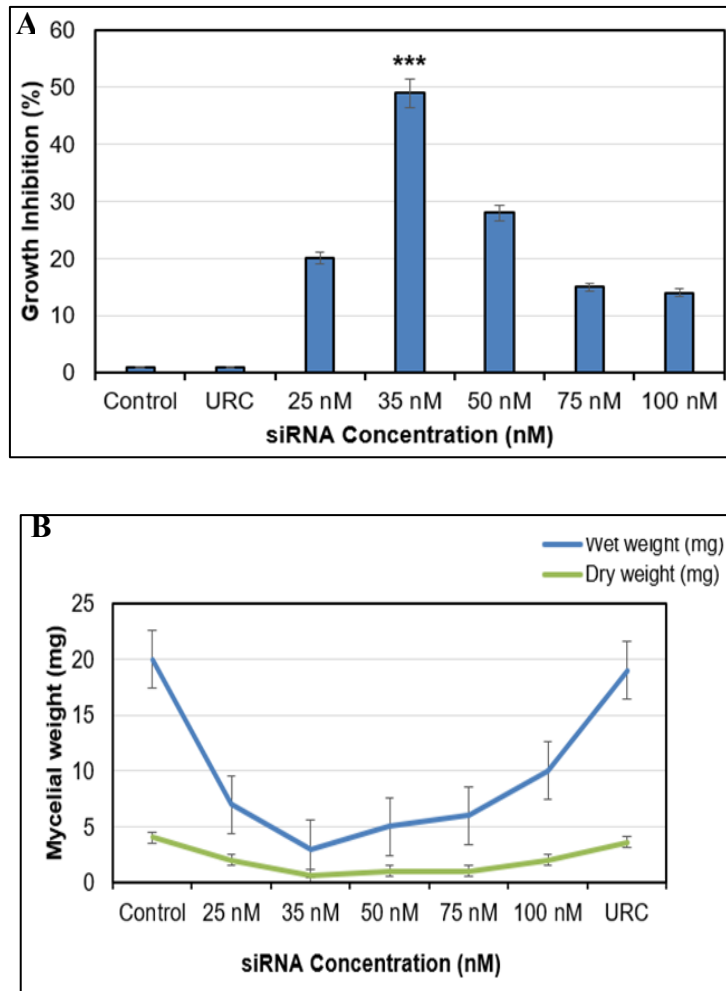


Figure 3.3 The effect of siRNA on fungal growth and morphology of *F. graminearum* on PDA medium. (A): Graph showing fungal growth inhibition. (B): Mycelial biomass (mg) post-siRNA treatment.

To evaluate the effect of *CRP* siRNA on sporulation and conidia germination in *F. graminearum*, the fungal conidia harvested from the culture medium were treated with siRNA at different concentrations. The number of germinating spores in the *CRP*-specific siRNA-treated samples were relatively lesser compared to controls. About 50% reduction was observed in 35 nM treated samples with no alteration even after increasing the concentration of siRNA. Further, it was also observed that there was a dramatic reduction in conidial germination in siRNA-treated germinating spores at 75 nM as compared to untreated control and 35 nM unrelated siRNA-treated control. The germinating conidia treated with different concentrations

of siRNA (10, 15, 25, 35, 50, 75, and 100 nM) showed notable inhibition of hyphal growth compared to control spores. Although the sporulation rate was significantly lesser, the treated samples produced a majority of short and broad deformed conidia. A drastic change in morphology with visible deformations such as irregular hyphal growth, bulged sac-like, and conglobate structures were observed at hyphal tips at 24 and 48 hpt as observed in (**Figure 3.4**).

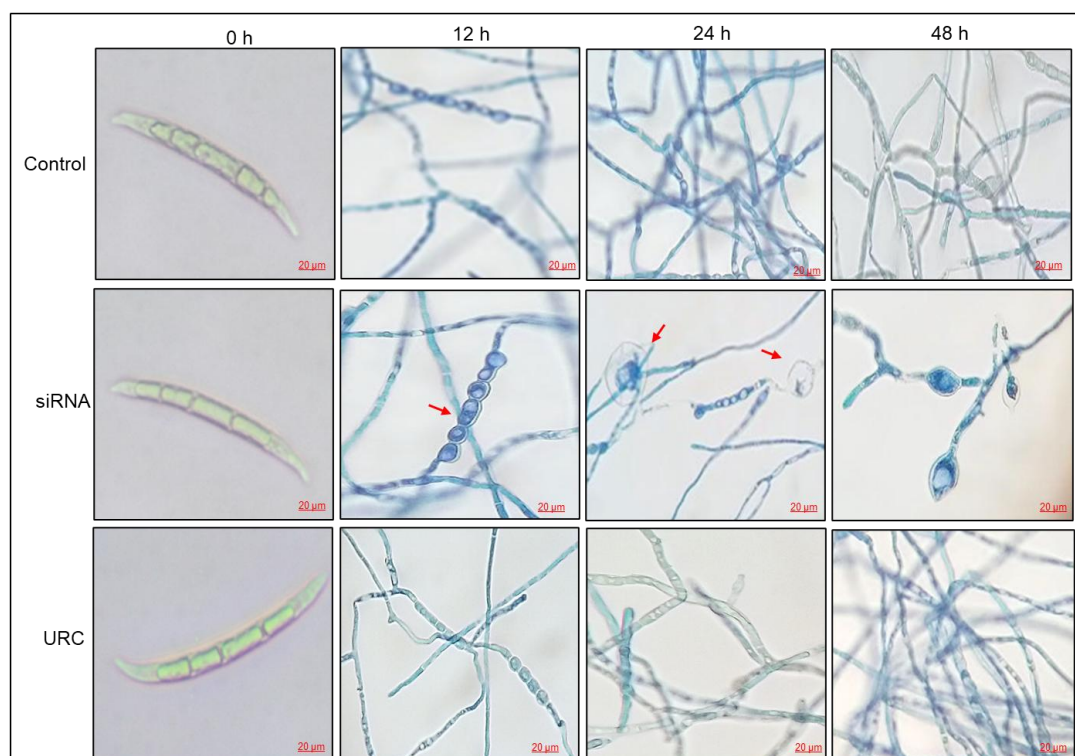


Figure 3.4 Effect of *CRP* specific-siRNA on *F. graminearum* sporulation and conidial germination in PDB medium at 6 hours post-germination. Magnification: 40x; bar: 20 µm.

3.3.4 Differential gene expression of *CRP*

The RT-PCR results carried out in both *in-vitro* at 48 h and *in-planta* at 5 dpt in *F. graminearum* and Wheat confirmed the down regulation of the *CRP* gene expression upon siRNA treatment in comparison to the control and unrelated control samples. As the incubation time progressed there was a gradual increase in mRNA levels at 72 hpi (data not shown) and the mRNA levels were similar to control and treated samples. The agarose gel images shown are (**Figure 3.5**) are the representation of the same experiment.

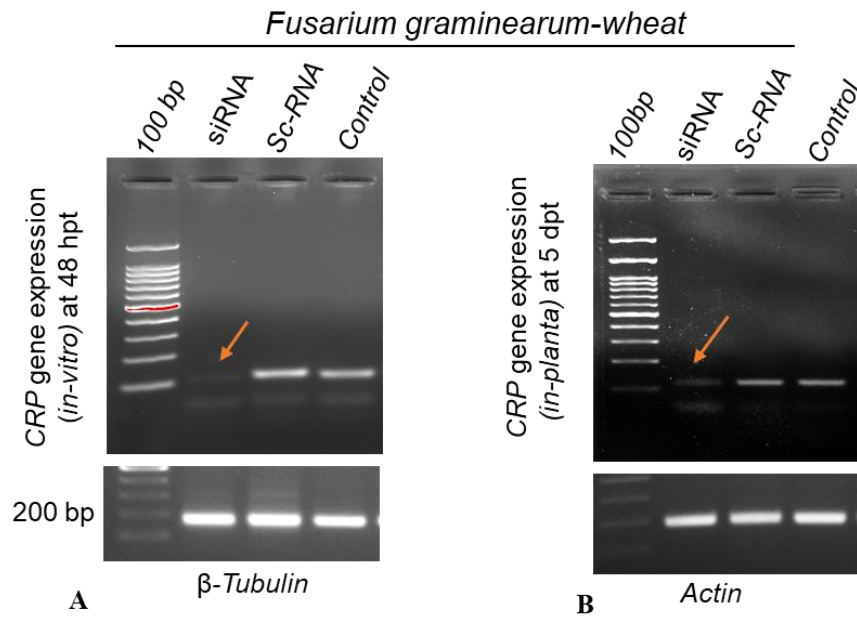


Figure 3.5 Reverse transcriptase (RT-PCR) based *CRP* gene expression in *in-vitro* and *in-planta* studies. (A): *CRP* gene expression in siRNA-treated and untreated *F. graminearum* at 48 hpt in *in-vitro*. (B) 5 dpt (*in-planta*).

3.3.6 Disease symptom evaluation in siRNA-treated wheat leaf and spikes

To assess whether the *F. graminearum*-*CRP* (*FgCRP*) gene was a suitable target for the SIGS-mediated plant protection strategy, the disease phenotype of leaves and spikes in siRNA-treated wheat plants challenged by *F. graminearum* were monitored in comparison to the controls. The controls comprised mock (autoclaved water)-treated followed by *F. graminearum*-pathogen challenged samples and unrelated control. The siRNA treatment reduced the fungal infection on leaves and FHB symptoms on spikes in comparison to controls. The average lesion size also reduced significantly in wheat leaves challenged with siRNA-treated *F. graminearum* in comparison to the controls which were challenged with *F. graminearum* alone (Figure 3.6 A-B-C).

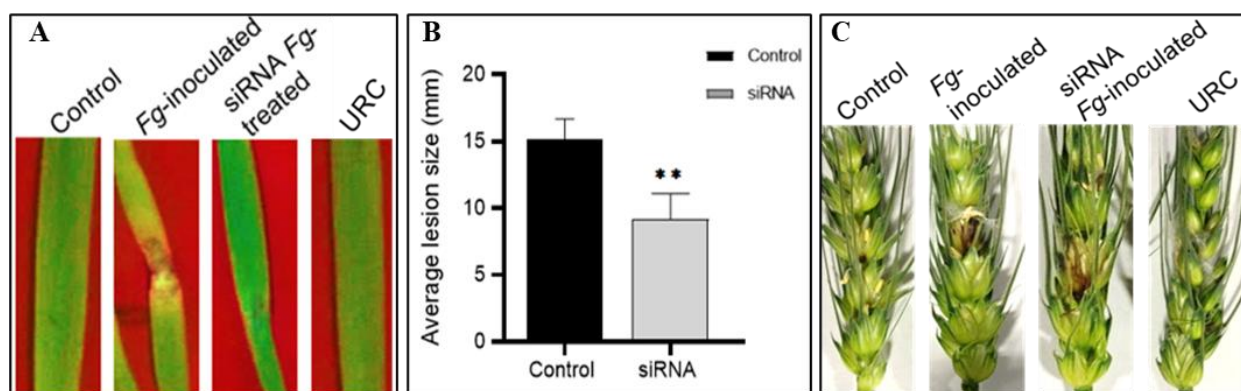


Figure 3.6 Pathogenicity assay of *F. graminearum* *CRP* specific-siRNA on wheat leaves and spikes (A-C). The graphical representation of quantification of lesion size of wheat leaves (B).

3.3.7 Analysis of the target gene (*CRP*) by qRT-PCR in *F. graminearum*

The gene expression studies using *CRP* gene-specific primers in axenic cultures indicated that there was a significant reduction in mRNA levels of siRNA-treated samples at 48 hpt of incubation as compared to untreated control. The axenic cultures of the phenotypically distinguishable siRNA-fed culture demonstrated a significant reduction in the *CRP* transcripts. Likewise, gene expression studies in wheat spikes (*in-planta*) revealed the down-regulation of the *CRP* gene in siRNA-treated wheat challenged by *F. graminearum* in comparison to the controls which were challenged with *F. graminearum* alone at 5 dpt indicating an effective silencing of the *CRP* gene in this study (Figure 3.7).

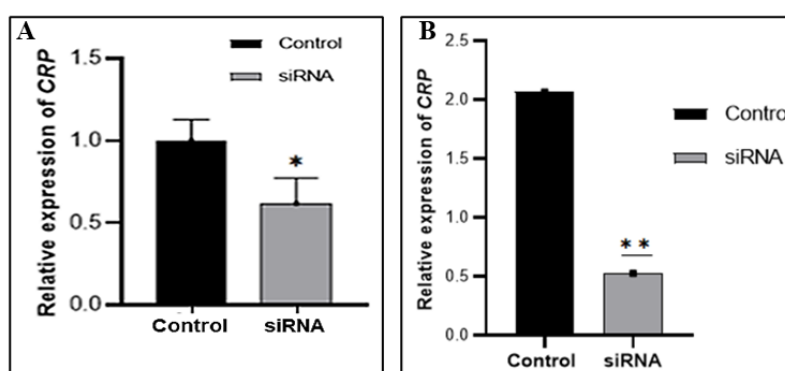


Figure 3.7 (A) qRT-PCR expression of *CRP* gene in siRNA-treated *F. graminearum* in PDB medium at 48 hpt. (B): Fold-change *CRP* gene expression in siRNA-treated wheat spikes at 5 dpt.

3.3.8 Effect of siRNA on Arabidopsis leaves

The extent of leaf infection was assessed by calculating the proportion of the leaf area affected by chlorotic lesions relative to the total leaf area, employing ImageJ software (Schneider et al., 2012). After 5 dpt, the necrotic lesions were prominently visible at the inoculation site in Arabidopsis leaves challenged with *F. graminearum* alone. The lesion sizes were significantly larger in *F. graminearum*-inoculated Arabidopsis leaves while the siRNA-sprayed leaves showed reduced infection as revealed by the smaller lesion size. The size of lesions was quantified and shown graphically. Similarly, RT-qPCR results revealed relatively low transcript levels of *CRP* gene in the siRNA-sprayed samples challenged with *F. graminearum* in comparison to the untreated control Arabidopsis leaves-challenged with the fungal pathogen as described in (Figure 3.8).

3.3.9 siRNA uptake assay, colony growth, and total biomass assays of *M. oryzae*

To check whether *F. graminearum* *CRP*-siRNA can knock down its homolog in *M. oryzae*, an ascomycetes hemibiotrophic pathogen of rice, The *in-vitro* treatment involved co-incubation of *M. oryzae* spore with *CRP*-specific siRNA in both solid and liquid media.

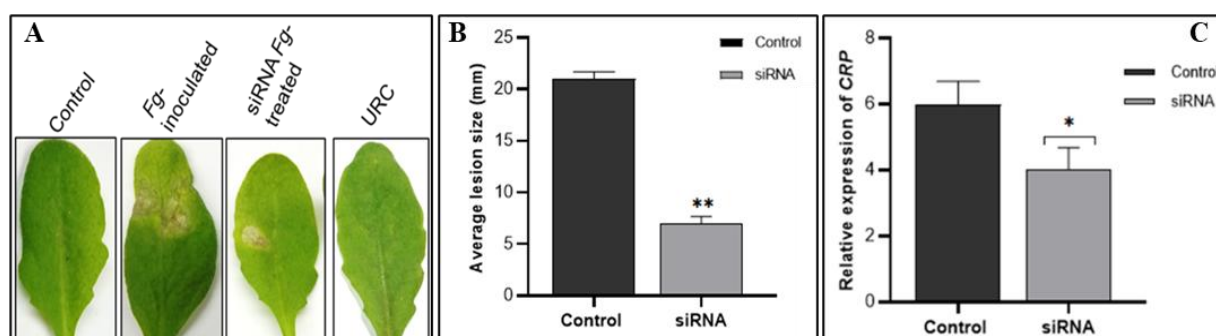


Figure 3.8 (A) Pathogenicity assay of spraying of *CRP* specific-siRNA on Arabidopsis leaves on lesion development by *F. graminearum* TN01. (B): The length of lesion on Arabidopsis leaves at 5 dpt. (C): The mRNA expression of the *CRP* gene post-siRNA treatment of Arabidopsis leaves

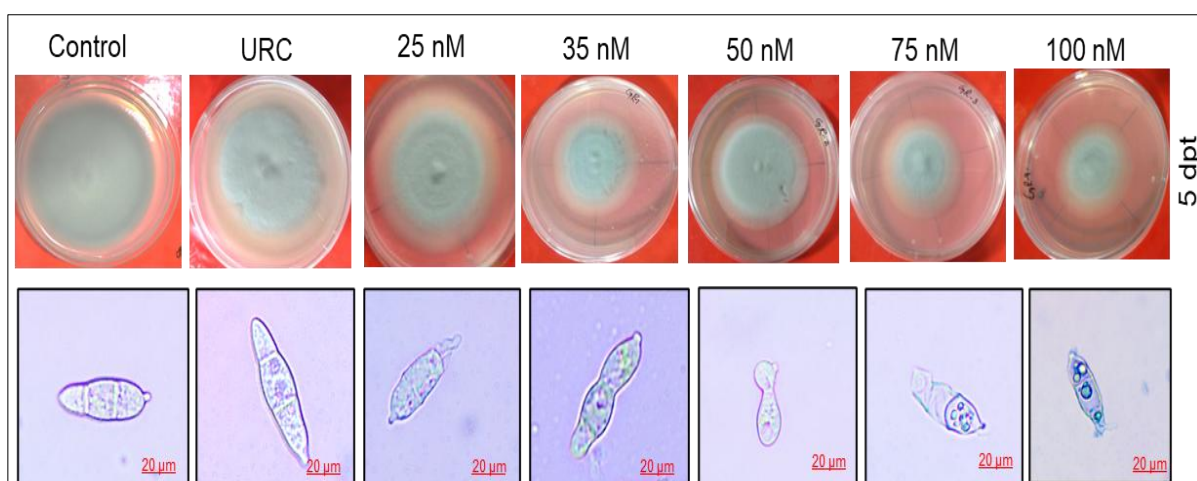


Figure 3.10 Effect of exogenous application of siRNA on mycelia growth of *M. oryzae* on PDA medium at 5 dpt. Colony growth and conidia morphology of *M. oryzae* at different concentrations of siRNA.

In addition, aerial hyphal development was significantly reduced in siRNA-treated *M. oryzae* containing PDA plates. Mycelia were sparsely developing in the plates containing siRNA-treated *M. oryzae* fungus producing concentric rings around the edges. The change in the size of the colony diameter was significant in siRNA-treated *M. oryzae* in comparison to the control (without siRNA) and the unrelated control (URC) at 5 dpt as shown in (Figure 3.10).

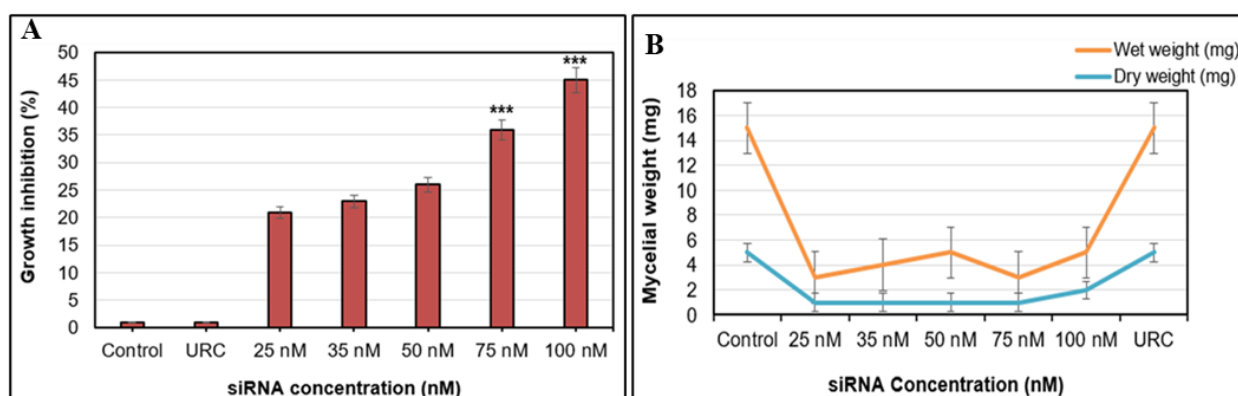


Figure 3.11 (A) Graphical representation of percent growth inhibition post-siRNA treatment. (B): Mycelial biomass (wet-weight and dry-weight) post-siRNA treatment.

Though a significant level of mycelial reduction was observed at 35 nM concentration in comparison to 50 nM, the level of reduction was very high at 75 nM followed by 100 nM. The growth inhibition induced by *CRP*-siRNA treatment showed a gradual increase in reduction starting from 25 nM concentration with the highest reduction at 100 nM concentration. Though the biomass reduced in all siRNA treated samples, a notable reduction in total biomass was observed in case of wet-weight of the mycelia compared to its dry-weight. The reduction in total biomass in *CRP* siRNA-treated samples in the case of dry-weight was found to be similar for the concentrations– 25 nM, 35 nM, 50 nM, and 75 nM. The graphs are visual representation in of growth inhibition and fungal biomass as shown in (Figure 3.11).

3.3.10 Conidial morphology of *Magnaporthe oryzae*

The spore morphology also varied drastically for all the *CRP*-gene specific siRNA treatments as shown against the specific concentration. Exogenous siRNA treatment of *M. oryzae* decreased the mycelial growth as visualized by measuring the diameter of the fungal colonies formed by inoculating siRNA-treated germinating conidia which were substantially smaller than the untreated control. Although the sporulation rate was much lower, the treated samples produced a higher proportion of short and broad deformed conidia. Likewise, the conidial germination and appressoria development was significantly reduced in a time-course study of siRNA-treated *M. oryzae* compared to the control and unrelated control (URC) as visualised in the microscopic images shown in (Figure 3.12).

3.3.11 Pathogenicity assay on rice leaves

To check whether similar *CRP*-siRNA-induced gene silencing as observed in *F. graminearum* in wheat and Arabidopsis, is also possible in a different pathosystem such as rice-*M. oryzae*, the siRNA-based experiment was carried out in rice plants as well. A 7 dpt, the necrotic lesions were prominently visible at the inoculation site in rice leaves challenged with *M. oryzae* alone. The

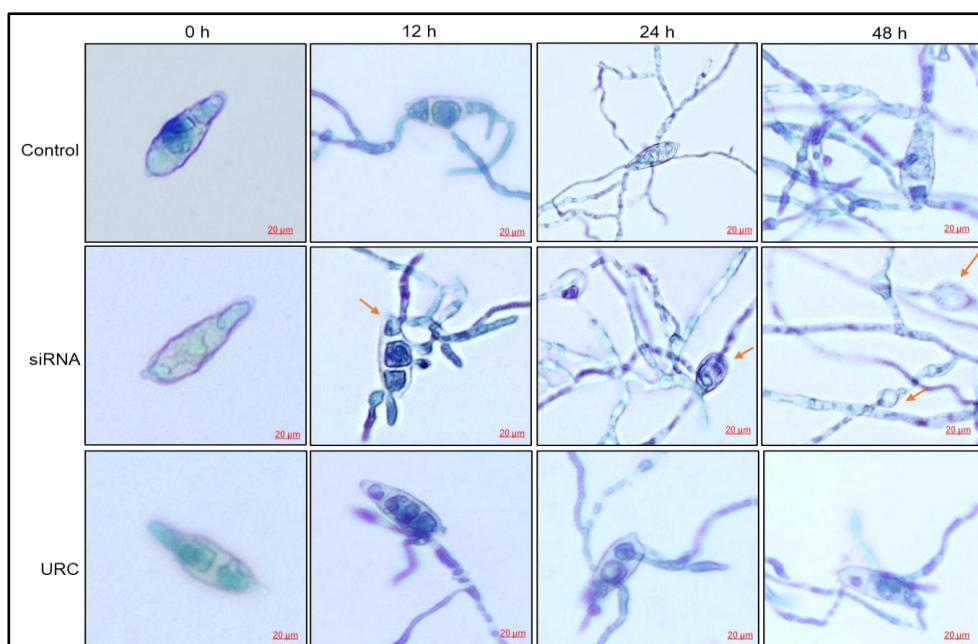


Figure 3.12 Conidia germination at different time points post-siRNA at 100 nM concentration. The conidial germination and mycelial formation of *M. oryzae* observed at 12, 24, and 48 hpt. Magnification: 40x, 20 µm.

lesion sizes were significantly larger in *M. oryzae*-inoculated rice leaves while the siRNA-sprayed leaves showed a significant reduction in infection as revealed by the smaller lesion size when quantified as shown graphically. Overall, the exogenous siRNA treatment resulted in lowering the level of fungal infection shown in (Figure 3.13).

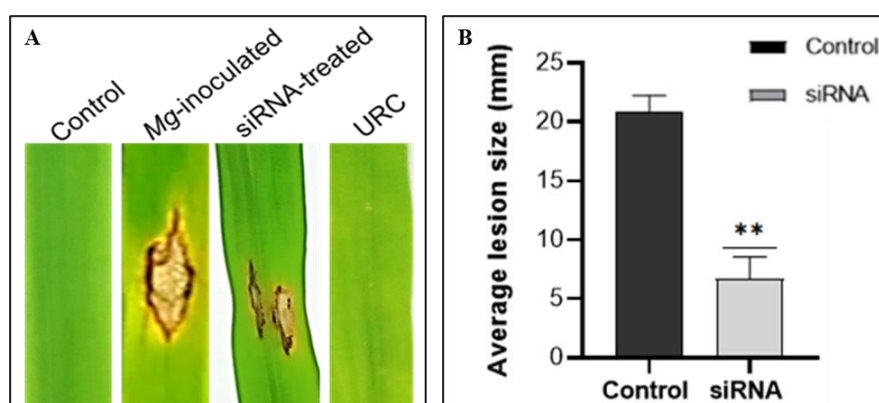


Figure 3.13 (A) Pathogenicity assay using *CRP* specific-siRNA treated *M. oryzae* conidia on rice. (A): Disease phenotype on rice with siRNA treated-*M. oryzae* spores at 7 dpt. (B): The mean lesion size of blast leaf spots on rice leaves at 7 dpt.

3.3.12 Relative gene expression of *CRP* by qRT-PCR.

The differential gene expression revealed that there was a significant downregulation of the *CRP* gene at RT-PCR level in both exogenously fed siRNA treated samples at 48 h and *in-planta* studies in rice at 7 days post siRNA treatment. Similarly, the *CRP* transcript levels in *M. oryzae* when checked by qRT-PCR, revealed that the relative transcript levels were significantly reduced in the siRNA-treated spores in-vitro at 48 h and siRNA-sprayed rice leaves at 7dpt in comparison to the control as shown in (**Figure 3.14**), confirming the silencing of the *CRP* gene via SIGS.

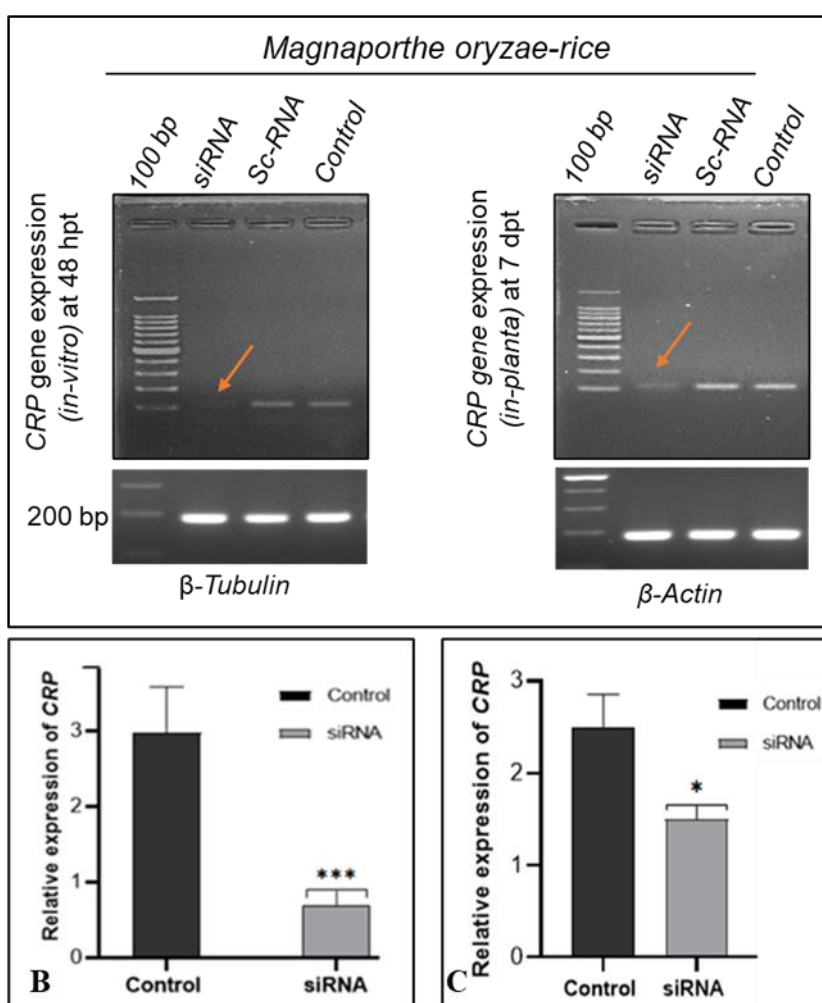


Figure 3.14 (A) *CRP* gene expression at RT-PCR level and by qRT-PCR in siRNA-treated and untreated *M. oryzae* at 48 hpt (*in-vitro*) (B) and 7 dpt (*in-planta*) (C). Fungal-specific β -Tubulin and plant-specific *Actin* gene expression in the gel image in both panels are shown as internal controls. Marker: 100 bpi.

3.4 DISCUSSION

In contrast to mammals, microbial pathogens and pests possess the ability to absorb environmental small RNAs (sRNAs), which are noncoding RNAs that maintain their RNA interference (RNAi) functionality (Khatri & Rajam, 2007; McEwan et al., 2012). This finding suggests that exogenous RNA can protect plants from infections. The RNAi method precisely silences a particular gene using a dsRNA molecule complementary to its target mRNA sequence (Wilson & Doudna, 2013). It has been shown by previous studies that two possible RNA uptake pathways are present and work in two different ways. The fungal cells take up the RNA, thereby inducing the fungal RNAi machinery directly. Similarly, the host/plant cells take up the RNA provided to them via liquid or solid media, thereby inducing the RNAi machinery of host cells (Kotch et al., 2016; Wang et al., 2016; Wang & Jin, 2017).

The SIGS provides an easy way to target certain genes in filamentous fungi. In the present study, SIGS was confirmed by gene expression studies of the *CRP* gene using gene-specific primers in not only *F. graminearum* but also in *M. oryzae*, another hemibiotrophic fungal pathogen. Application of dsRNA on potato leaves resulted in the silencing of the developmentally critical genes in the oomycete pathogen, *Phytophthora infestans*, such as *PiGPB1*, *PiCut3*, *PiHmpl1*, and *PiEndo3*. A significant reduction in the expression of the respective gene transcripts was also observed, mitigating the late blight disease of potato by SIGS (Kalyandurg et al., 2025). Earlier studies also revealed that the dsRNA could be actively taken up by the cysts and sporangia of *P. infestans*, however, the specific mechanism of the dsRNA uptake is still to be investigated (Qiao et al., 2021).

In plant pathogenic fungi and other eukaryotes, for the control of vegetative growth, sexual or asexual reproduction, and infection-related morphogenesis, the cAMP-PKA has been advocated as the critical pathway and is generally conserved (Turra et al., 2014; Hu et al., 2014; Voisey et

al., 2016; Yang et al., 2016). Also, it is important to note that cAMP signaling is crucial for combating the hemibiotrophic fungal pathogens caused infections such as *F. graminearum* and *M. oryzae*, involving multiple steps including conidial germination, appressorium formation, penetration peg formation, and invasive hyphal growth (Wilson & Talbot, 2009), and production of mycotoxins, phytotoxins, and other secondary metabolites, such as biosynthesis of ethylene in *V. dahliae* and generation of AT toxin in *Alternaria alternata* (Tzima et al., 2010; Park et al., 2016).

The siRNA treatment of *F. graminearum* and *M. oryzae* samples resulted in a significant decrease of mRNA transcripts of *CRP*, at 48 h of treatment, as expected in the case of SIGS when compared to untreated controls. Further, RTq-PCR-based gene expression studies also showed significant down-regulation of the *CRP* gene in both *in-vitro* and *in-planta* compared to the untreated control samples in both pathogens, thereby inhibiting fungal infection and growth. Previous studies carried out in banana wilt causing *F. oxysporum* led to the partial silencing of the dicer-like 2 (*DCL2*) gene post-dsRNA exogenous application by *in-vitro* at 72 hpt (Jayasekara et al., 2025).

Our results demonstrated that targeting the key component of the cAMP regulatory gene (*CRP*) by exogenous application of siRNA revealed a clear distinction between the siRNA-treated conidia and control, untreated conidia when visualized under a light microscope. The effect of *CRP*-siRNA treatment on both *F. graminearum* and *M. oryzae* conidial cultures led to the alteration of the conidial morphology, reduction in mycelial growth, sporulation, and total biomass in liquid cultures supplemented with the siRNA, indicating the knockdown of the *CRP* gene in those fungal samples. Both axenic cultures and *in-planta* studies with exogenous treatment of *CRP*-siRNAs showed reduced mycelial growth, conidial germination, and total biomass of both *F. graminearum* and *M. oryzae*. As the incubation time progressed, there was a gradual increase in mRNA levels at 72 hpi (data not shown), and the mRNA levels were similar

to control and treated samples, suggesting the rapid adaptability and cellular stability to the exogenous treatment in fungi.

The *in-planta* studies also revealed significant down-regulation of *CRP* gene transcript abundance in SIGS using *CRP*-specific siRNAs, followed by inoculation of leaves of wheat at 5 dpt and rice at 7 dpt with the fungal pathogens, *F. graminearum* and *M. oryzae*, respectively. Likewise, the mycelial growth on the PDA plates supplemented with siRNA resulted in a significant decrease at 5 and 7 dpt. One such study performed in *M. oryzae* by applying the artificial *MoAPI*-specific siRNA *in-vitro* and *in-planta* resulted in the silencing of the *API* gene. The uptake of the siRNA led to the inhibition of mycelial growth, spore abnormality, restricted appressoria, and compromised pathogenicity in comparison to the control (Guo et al., 2019). Our findings were in accordance with the previous studies performed by Bhagta et al. (2023) wherein it was demonstrated that exogenous application of dsRNA *in-vitro* on *Venturia inaequalis* resulted in the reduced growth of mycelia and sporulation along with the significant down-regulation of *VICE12* expression levels in treated conditions compared to the control. Similarly, earlier studies have indicated that gene silencing induced by siRNA, specifically targeting the ornithine decarboxylase (*ODC*) gene, which is crucial for polyamine biosynthesis in *A. nidulans*, highlights the essential role of cellular polyamines in the normal development of germ tubes and mycelia (Khatri & Rajam, 2007).

The morphology of conidia of *F. graminearum* showed visible distortions like the deformed mycelia with sac-like crooked conglobate structures visible at the hyphal tips at 24 and 48 hpt. In addition to this, we observed a significant reduction in the sporulation, accompanied by irregular conidial germination and small-sized conidia in siRNA treated condition in comparison to the control. Our findings were consistent with the earlier research studies (Mumbanza et al., 2013), which demonstrated that applying dsRNA *in-vitro* led to different levels of spore germination

inhibition by triggering the silencing of target genes in two primary banana-infecting phytopathogens, *Fusarium oxysporum* and *Mycosphaerella fijiensis*. This makes them essential targets for RNAi-induced resistance in bananas against these fungal pathogens. Similarly, the exogenous application of *SsMNO1*-specific siRNA in *Sclerotinia sclerotiorum* resulted in inhibitory effects on hyphal growth, sclerotial development, and virulence *in-vitro* as well as in *Arabidopsis (in-planta)* (Han et al., 2024).

The SIGS performed using *CRP*-siRNA in a concentration-gradient experiment revealed that the effective concentration for *CRP* gene knockdown was at 35 nM in-vitro cultures of *F. graminearum* as well as *M. oryzae* supplemented with the siRNAs compared to control samples. Similarly, earlier studies showed that siRNA molecules, at an optimal concentration of 25 nM, were effectively absorbed by germinating fungal spores from the culture medium (Khatri & Rajam, 2007), and the results were in agreement with our findings, indicating significant silencing of *CRP* gene expression at 35 nM concentration.

The pathogenicity assay of *CRP* siRNA-treated *F. graminearum* on the leaves and spikes of wheat, and leaves of *Arabidopsis* via SIGS, also resulted in a significant reduction in lesion size post-siRNA treatment at 5 dpi. This finding was in accordance with the results of a previous study performed on the *F. graminearum* *FgPMA1* gene, where the application of the *FgPMA1* specific-dsRNA resulted in a significant decrease in the disease lesion size on the leaves, coleoptiles, and the spikes of wheat along with reduced colony growth rate, deformed mycelia and the loss of sporulation activity. The study revealed that there was a significant decrease in the pathogenicity (Wu et al., 2023). Our results also back the idea that fungal RNAi-related genes in *F. graminearum* critical role in pathogenicity and/or virulence as reported previously (Gaffar et al., 2019; Liu et al., 2024). These findings are in line with previous study revealing that the two Dicer-like proteins (*DCL1* and *DCL2*) of the fungal pathogen *Botrytis cinerea* play a crucial role

in disease development (Wang et al., 2016). The *CRP* gene was selected as the target gene of interest for silencing by RNA interference as this gene is one of the critical gene for *F. graminearum* virulence and pathogenesis (Li et al., 2018). Exogenously applied siRNA significantly reduced infection by *F. graminearum* on siRNA-treated wheat spikes, highlighting its potential to target the fungal infection in environmental or agricultural systems. Exogenous siRNAs demonstrate a capacity to impede mycelial growth in fungal cultures, a phenomenon that occurs independently of DCL activity in the target fungus (Wang et al., 2016, Werner et al., 2020). Overall, the results suggest that the silencing of the cAMP regulatory gene by siRNA could be used to protect wheat from *F. graminearum* infection as well as rice from *M. oryzae*. Together, our results indicate that silencing the *F. graminearum* cAMP pathway gene, *CRP*, using SIGS efficiently increases plant disease resistance towards hemibiotrophic fungal pathogens, *F. graminearum* and *M. oryzae*. Therefore, efficient uptake of *CRP*-siRNA by fungal infection hyphae could result from the efficiency of the SIGS setup as well as the sequence-specific gene knock-down, thereby enabling SIGS as a highly adaptive, more effective approach to target diverse pathogens with multiple genes that are essential for virulence or pathogenicity.

Chapter. 4

Comparing compatible and incompatible wheat-*F. graminearum* interactions for differentially expressed metabolites and validation through gene expression studies

4.1 INTRODUCTION

Fusarium head blight (FHB) caused by the fungal pathogen, *F. graminearum*, is a serious concern in wheat and other cereal crops worldwide (Bai & Shaner, 2004; Goswami & Kristler, 2004). FHB disease causes significant economic losses due to reduced yields and degraded grain quality, thereby rendering the produce unfit for consumption (Goswami & Kristler, 2005). The grains from the *F. graminearum*-infected spikes appear discolored, tomb-like, or shrivelled, chaffy with low test weight and poor germination (Bai and Shaner, 1994). The typical FHB incidence on spikes starts at anthesis with the central spikelet showing necrosis, followed by bleaching and occlusion of the conducting tissue (Mandalà et al., 2019). Previous studies demonstrated that trichothecenes produced by *Fusarium* species are potent mycotoxins with a role in virulence (Harris et al., 1999). The fungus-infected grains are infested with mycotoxins, including trichothecenes, which pose serious concerns to humans and cattle (Chen et al., 2019; Ferrigo et al., 2016). The mycotoxins reported to be associated with *F. graminearum* infection in wheat are zearalenones (ZEA) and trichothecenes with several chemical forms, such as the type B trichothecenes (TCT-B), deoxynivalenol (DON), its 3-acetylated (3-ADON) or 15-acetylated derivatives (15-ADON), and nivalenol (NIV) (McCormick et al., 2011).

An evolutionary arms race between the host and its adapted pathogen controls the defenses in the host and virulence in the pathogen (Hu et al., 2022). Moreover, timely recognition and activation of plant defenses appear to control the outcome of the host-pathogen interaction. It signifies the importance of defense signaling in regulating plant responses mediated by the interacting host and pathogen components (Ali et al., 2024). The complementary gene-for-gene interaction between the resistance (R) gene from the host and the avirulence (Avr) gene from the pathogen results in an incompatible (resistance) interaction. In contrast, the lack of a complementary gene-for-gene interaction between a host and its adapted pathogen leads to a 'compatible' (susceptible)

interaction (Bhosle et al., 2021). On the other hand, when resistance (R) genes interact with avirulence (Avr) genes in an 'incompatible' manner, it triggers a hypersensitive response (HR) and activates defense signaling to inhibit the pathogen's growth within the host (Balint-Kurti, 2019; Nim Chuk et al., 2001).

Though emerging evidence indicated the production of a repertoire of molecular determinants and metabolites from wheat-*F. graminearum* interactions, our understanding of the resistance-related (RR) metabolites are limited. Metabolomic studies provide a high throughput screening to identify resistance-associated biomarker metabolites which have the advantage of integrating diverse genetic backgrounds for breeding for complex quantitative trait loci (QTLs) controlling FHB resistance in wheat (Gauthier et al., 2015). We hypothesize that metabolomic analyses between a mock (water-treated) and a pathogen-inoculated, susceptible, and resistant cultivars may reveal the potential metabolite perturbations between compatible and incompatible interactions by comparing constitutive and induced defense responses against *F. graminearum* in wheat.

Analyzing the metabolomes of plants in response to fungal pathogens facilitates not only detection of the defense-related compounds but also enhances our understanding of the mechanisms underlying plant-pathogen interactions (Kumar et al., 2015, 2016). Metabolomics has emerged as a powerful tool to study plant metabolic responses against both biotic and abiotic stresses. Alterations in plant metabolism play a central role in determining the outcome of plant's response to those stress conditions. A large-scale detection and quantification of the metabolites altered by a specific condition is achievable using non-targeted metabolomics (Allwood et al., 2008).

Utilizing GC-MS, previous studies carried out to analyze the metabolic profiles of wheat spikelets from the cultivars, Roblin (susceptible) and Sumai3 (resistant), revealed the altered metabolites

(Hamzehzarghani, et al., 2005, 2008). Similarly, non-targeted metabolomics coupled with proteomics was used to detect *Fhb1*-mediated resistance responses in wheat for high-throughput screening of wheat and barley (Gunnaiah et al., 2012). It was also demonstrated that identifying host resistance factors can assist in combating FHB susceptibility in wheat (Warth et al., 2015). In this study, metabolomic profiling of the wheat genotypes: resistant (Nobeoka) and susceptible (Sonalika) challenged with *F. graminearum* strain TN01 were compared in relation to their mock-controls to detect resistant-related (RR) compounds by adopting non-targeted metabolomics approach using GC-MS analysis at 48 hpi and the findings are presented.

4.2 MATERIALS AND METHODS

4.2.1 Plant and fungal culture conditions

The wheat genotypes– FHB resistant, Nobeoka (Atanassov et al., 1994), and FHB-susceptible Sonalika (Kumar et al., 2021) used in the study were obtained from the National Bureau of Plant Genetic Resources (NBPGR), New Delhi. Wheat seeds were surface-sterilized as described by Fatima et al (2024). The plants were grown at the plant culture facility of the University of Hyderabad (UOH) (Telangana, India) which was maintained at 23 ± 2 °C with 16 h of light and 8 h of dark.

The *F. graminearum* TN01 strain with Microbial Type Culture Collection (MTCC) Accession No. 13568, previously isolated and identified from wheat fields of the IARI regional station Tamil Nadu, India (Fatima et al., 2024) was used for inoculation. The fungal strain was grown on PDA (Himedia India) medium at 25 °C for 7 days. For culturing spores, 5 activated strain blocks (6 mm in diameter) were placed in a 200 mL flask filled with 100 mL of sterilized mung bean broth with shaking at 200 rpm at 25 °C for 3 days (Makandar et al., 2012).

4.2.2 Plant inoculation and sample collection

Using a hemocytometer, the conidial suspension was prepared in sterile water by adjusting the spore concentration to 10^5 conidia/mL. At the mid-anthesis stage, inoculation of wheat spikelets was carried out as described by (Feng et al. (2018)). Each spikelet was inoculated with 10 µL of a fungal spore suspension with a concentration of 1×10^5 conidia/mL. Similarly, mock treatment of control plants was performed by pipetting autoclaved distilled water. To ensure high moisture levels, inoculated plants were covered with water-sprayed polyethylene zip-lock bags, which were removed 48 hpi. The samples were collected in three replicates with each replicate containing

three pairs of inoculated and mock-treated spikelets separately from a total of six spikes per treatment. The samples were snap-frozen in liquid nitrogen and stored at -80°C .

4.2.3 Chemicals and reagents used in the study

Methanol and water of HPLC grade, chloroform and acetone of analytical grade, ultra-pure ribitol, pyridine, methoxylaminehydrochloride, N, O-bis (trimethylsilyl) trifluoro acetamide (BSTFA, 98%) with trimethylchlorosilane (TMCS, 1%) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Deoxynivalenol (DON) for anti-fungal testing was also purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

4.2.4 Sample preparation and metabolite extraction

For the extraction of metabolites, a protocol given by Lisec et al. (2006) was followed. It is as follows: 100 μL of ribitol (0.9 mg/mL) was added for measurement after combining 100 mg of plant extract with 1.4 mL of methanol. The mixture was extracted by incubating at 70°C for 15 min followed by centrifugation at $11,000 \times g$ for 10 min. After transferring the supernatant to a reaction tube, 1.5 mL of water and 750 μL of chloroform were added. The top phase was dried under vacuum using SpeedVac at $2200 \times g$ for 15 min and stored at -80°C .

Samples were derivatized by shaking them for 10 min with 40 μL of pyridine containing 20 mg of methoxyamine. Bis-trimethyl-N-trifluoroacetamide (BSTFA) was added following the incubation of the mixture at 37°C for 90 min. The contents of the samples were briefly vortexed and the samples were incubated at 37°C for 1 hr. The residue was derivatized using a two-step procedure. First, 80 μL of methoxyamine (20 mg/mL in pyridine) was added to the vial and incubated at 30°C for 90 min. Second, 80 μL of BSTFA (1% TMCS) was added to the vial and maintained at 70°C for 60 min. Subsequently, 5 μL of the filtered sample was used for metabolite

analysis using gas chromatography-mass spectrometry (GC-MS) at NovelGene Technologies, University of Hyderabad, India.

The GC-MS analysis used an Agilent 7890A/5975C GC-MS system (Agilent, USA) with an Agilent HP -5MS capillary column (30 m $\times$ 0.25 mm, 0.25 μ m film thickness). Helium served as carrier gas at 1 mL min⁻¹. Samples of volume 1 μ L were injected into the column at a split ratio of 1:3. The GC oven temperature was maintained at 70 °C for 2 min, increased to 290 °C at a rate of °C min⁻¹, and held for 3 min. The temperatures of the injection port, transfer line, and ion source were set at 300 °C, 280 °C, and 250 °C, respectively. A mass scan range of 50–600 m/z at 20 scans s⁻¹ was employed. Mass spectrometric data were acquired using real-time analysis software in the GC-MS system (Agilent, USA). A set of quality control (QC) samples was prepared by mixing equal volumes of the extracts from all samples. Each QC sample was of the same volume as the original samples and injected at regular intervals throughout the analytical run to provide data for assessing repeatability.

4.2.5 Computational data processing

The raw mass spectrometric (MS) data (wiff. scan files) was converted to MzXML MZmine 2.53 (<http://mzmine.github.io/>) files by Proteo Wizard MS Convert and imported into XCMS software (Scripps Research, La Jolla, CA, USA). Variables having more than 50% of the non-zero measurement values in at least one group were submitted for the following analyses. Compound identification was performed by comparing the accuracy m/z value (<25 ppm). Mass detection was conducted, chromatograms were constructed, and peak alignment was achieved utilizing the respective algorithms available in MZmine 2.53.

The statistical data analysis was carried out by both univariate and multivariate methods. Univariate methods include fold-change analysis, t-tests, and volcano plots while multivariate methods used were principal component analysis (PCA) and metabolic pathway analysis. The

metabolic pathway diagrams were obtained with MetaboAnalyst 5.0. <https://www.metaboanalyst.ca> (Pang et al., 2021).

Partial Least Squares—Discriminant Analysis (PLS-DA) facilitated in identifying sample correlations and key variables. The variable importance in the projection (VIP) values for each of the variables was calculated using the OPLS-DA model and the values with VIP >1 and p-value <0.05 were used to screen significantly changed metabolites. Heatmaps for data visualization were created using MetaboAnalyst 5.0 in several ways under hierarchical clustering. Pathway impact analysis employed KEGG Mapper to compare KEGG items to pathway maps.

4.2.6 Metabolite detection and statistical analysis

The peak area of each sample was divided by the median value of the compounds detected across all samples, and the relative value was obtained for data analysis. Changes in the metabolite composition were compared using a heatmap and a p-value of <0.05 was considered statistically significant. The log₂ fold-change was used to normalize the data to a standard normal distribution, and a comparison between treatments was performed by student t-test. The data was analyzed using the analysis of variance (ANOVA) technique to evaluate overall significance followed by an LSD test at a 5% probability level to compare treatment means. Based on a standardized data matrix, a correlation matrix of the variables was estimated for principal component analysis (PCA). The KEGG IDs were derived for the metabolites detected through the sample analysis and the findings were exported.

4.2.7 RNA isolation, cDNA preparation

The total RNA from the plant samples was isolated using an RNA isolation kit (RNAiso Plus; Takara). Following treatment with RNase-free DNase (Takara) and ethanol precipitation, cDNA was synthesized using Superscript II (TAKARA). The cDNA was prepared from 1 µg of total RNA using the RT-PCR in a 20 µL reaction. The oligonucleotides used for gene expression studies are listed in (Table 4.1).

4.2.8 Gene Expression analysis by Real-time quantitative PCR (qRT-PCR)

The Real-time quantitative qRT-PCR was performed in a volume of 10 μ L. The 10 μ L reaction mixture was composed of 5.2 μ L of 1 $\times$ SYBR mix (TB Green Premix Ex Taq II; Takara), 0.5 μ L of 0.25 μ M primers, 1 μ L of 1:5 diluted cDNA derived from reverse transcription, and 2.8 μ L of water. The PCR amplification was carried out using a step-one Real-time PCR system (Applied Biosystem, Foster City, CA, US) under the following conditions: 95 $^{\circ}$ C for 5 min, followed by 40 cycles of 95 $^{\circ}$ C for 20 s, 60 $^{\circ}$ C for 20 s, and 72 $^{\circ}$ C for 20 s, with a plate read at 80 $^{\circ}$ C for 15 s. A melting curve was performed to determine the specificity of each PCR primer. The mRNA abundance of *PR1*, *PDF1.2*, *TRI5*, and *TRI6* genes relative to β -*Tubulin* as an internal control was checked by the $2^{-\Delta\Delta CT}$ method (Livak and Schmitt, 2003). The data obtained from qRT-PCR were subjected to the student's t-test, and a p-value of less than 0.05 was considered significant. Three independent samples (biological replicates) were used.

Table 4. 1 The list of primer sequences of mycotoxin and defense-related genes and internal controls used for qRT-PCR along with their expected amplicon size and annealing temperatures.

S. No	Name	Primer sequence 5'-3'	Amp Size (bp)	Annealing Temp ($^{\circ}$ C)
1	<i>TRI5</i>	F: TCTTAACACTAGCGTGCGCCTTCT R: CATGCCAACGATTGTTTGGAGGGA	110	59 $^{\circ}$ C
2	<i>TRI6</i>	F: TAACCACATCGTCGGGACTG R: GCCGACTTCTTGCAGGTCTT	100	53 $^{\circ}$ C
3	<i>PR1</i>	F: GGACTACGACTACGGCTCCA R: GGCTCGTAGTTGCAGGTGAT	250	53 $^{\circ}$ C
4	<i>PDF1.2</i>	F: CGGTTCTGGTTTGGCTTCTA R: TTGCTTTGGTTTCTGTAATGGA	250	48 $^{\circ}$ C
5	β - <i>Tubulin</i>	F: ATCTCGAGCCCGGTACCATGG R: CTCGGTGTAAATGACCCTTGCC	110	57 $^{\circ}$ C

4.2.9 Antifungal activity of DON

Deoxynivalenol DON (Cat. No: D0156, Sigma) was dissolved in water at a concentration of 1000 ppm (25 mg/L) and stored at 4 °C. *F. graminearum* TN01 spores were treated with different concentrations of DON. Different concentrations of 0.1, 1, 1.5, 2, 2.5, and 3 ppm were used for the treatment of *F. graminearum*. The Petri plates with PDA medium inoculated with *F. graminearum* were incubated at 26 °C for 6 days. The radial growth inhibition was assessed for 6 dpt with DON and visual images of fungal growth were recorded for 6 days. Experiments for all control and treated samples were repeated thrice.

4.3 RESULTS

4.3.1 Analysis of non-targeted metabolomic data

To detect metabolomic changes induced by the fungal treatment, metabolite profiles of the two wheat genotypes, Nobeoka (FHB-resistant) and Sonalika (FHB-susceptible) challenged with *F. graminearum* strain TN01 were compared at 48 hpi. A comprehensive analysis of all the metabolites underlying wheat-*F. graminearum* interaction revealed both constitutive and induced metabolites as shown in the Venn diagram (**Figure 4.1**). Comparison between mock (water)-treated and *F. graminearum*-inoculated wheat spikes of Nobeoka and Sonalika showed both shared or common metabolites as well as distinct metabolites between the two wheat genotypes following *F. graminearum* infection. Several differentially accumulated metabolites (DAMs) were detected in the wheat samples comprising the controls (NC: Nobeoka Control, SC: Sonalika Control) and *F. graminearum*-inoculated treatments (NT: Nobeoka Treated; ST: Sonalika Treated). A total of 809 metabolites were detected across all combinations, including 61 (NC), 20 (NT), 20 (NC vs NT), 15 (SC), 21 (ST), 14 (SC vs ST), and 8 (NT vs ST).

A bar diagram showing both significantly altered metabolites and non-significant metabolites detected in *F. graminearum*-challenged and control wheat samples are presented in (**Figure 4.2**). Pair-wise comparison between different treatments (ST vs NT, SC vs ST, and NC vs NT) revealed 182 up-regulated and 194 down-regulated metabolites in ST vs NT treatment. While 211 and 188 metabolites were identified as up-regulated and down-regulated, respectively, in SC vs ST, a relatively higher number of up-regulated (225) and down-regulated (289) metabolites were detected in NC vs NT. The metabolites detected in the wheat-*F. graminearum* interaction were categorized into various groups of compounds comprising alkaloids, terpenoids, phenols, flavonoids, amino acids and their derivatives, lipids, nucleotides and their derivatives, phenylpropanoids, saccharides and alcohols, organic acids and their derivatives, steroids and their

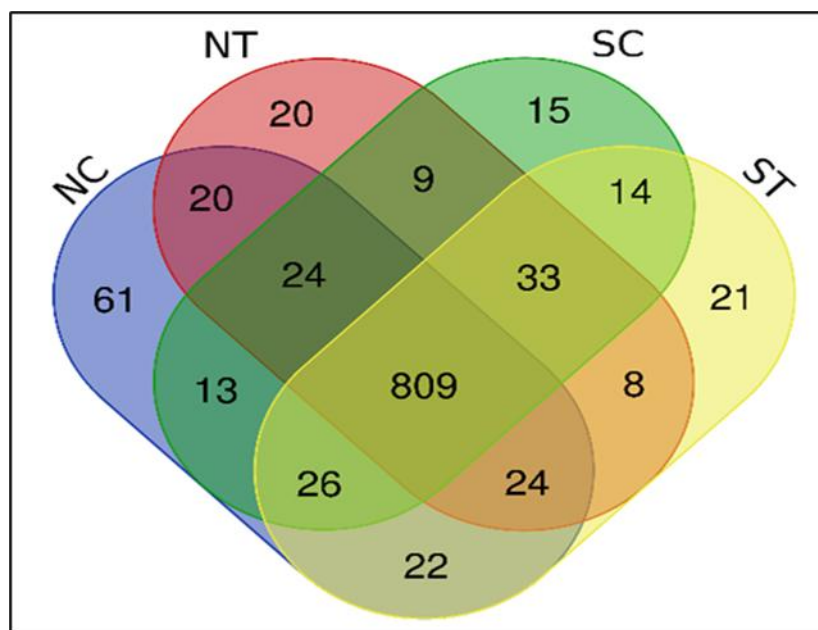


Figure. 4.1 Venn diagram showing distinct and common metabolites detected by Gas chromatography-mass spectrometry (GC-MS) in the susceptible (Sonalika) and resistant (Nobeoka) wheat genotypes challenged with *Fusarium graminearum* TN01 at 48 hpi.

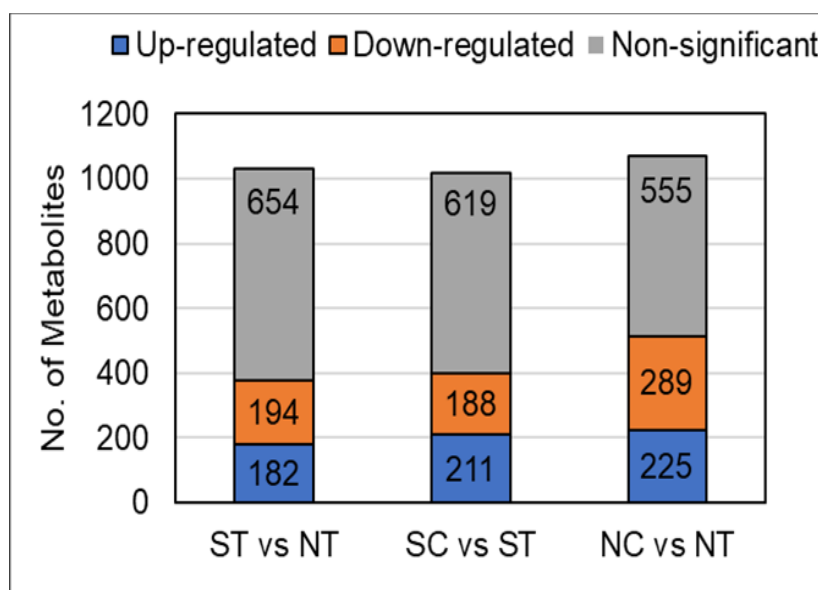


Figure 4.2 Bar graph representing significantly up-regulated and down-regulated metabolites along with non-significant metabolites detected in *F. graminearum*-challenged and mock (water)-treated wheat samples in ST vs NT, SC vs ST, and NC vs NT treatments.

derivatives, vitamins, quinonoids, and other classes as shown in (**Supplementary Table 4**). The relative proportion of various metabolite classes observed between *F. graminearum*-challenged

Nobeoka (Resistant) and *F. graminearum*-challenged Sonalika (Susceptible) in comparison to controls is presented in (Figure 4.3 A) and (Figure 4.3 B).

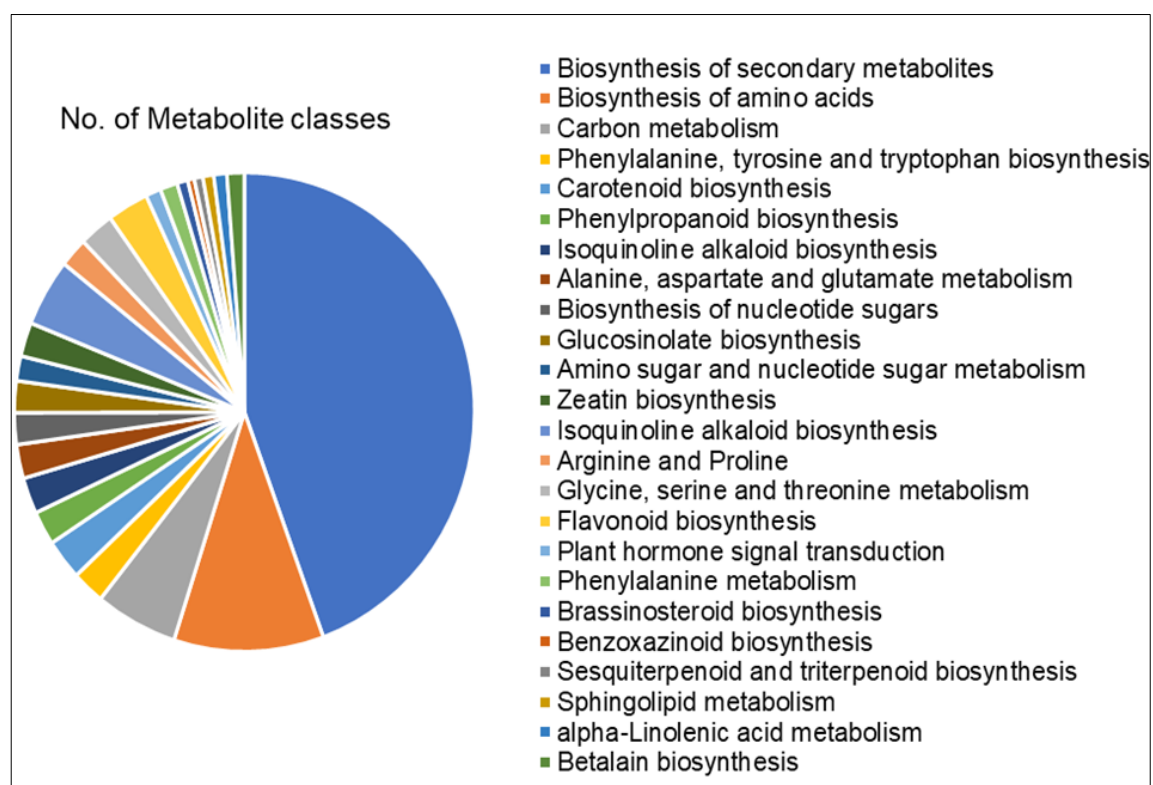


Figure 4.3 (A) Pie charts represent the categorization of the metabolites detected in wheat spikes challenged with *F. graminearum* TN01 by GC-MS analysis at 48 hpi. Metabolite groups identified in Nobeoka (NC vs NT).

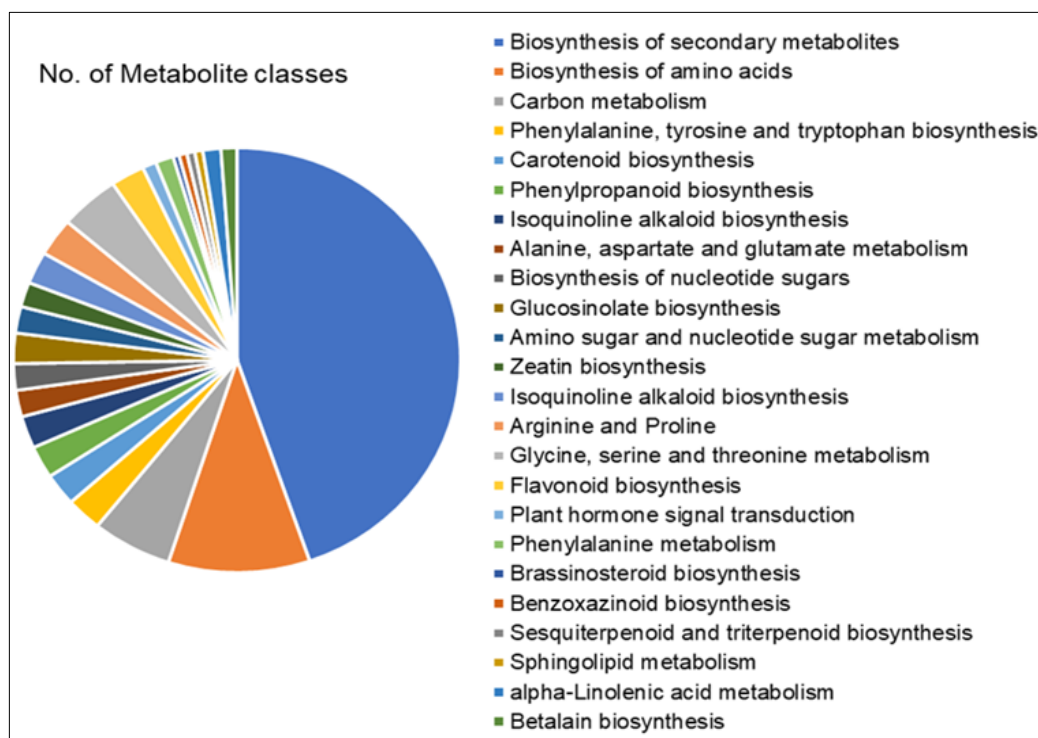


Figure 4. 3 (B) Pie charts represent the categorization of the metabolites detected in wheat spikes challenged with *F. graminearum* TN01 by GC-MS analysis at 48 hpi. Metabolite groups identified in Sonalika (SC vs ST).

4.3.2. Multivariate and principal component analysis

To gain a global picture of the distinct and common metabolites, a multivariate non-parametric statistical analysis was performed. Principal Component Analysis (PCA) was performed to assess the extent of variation among identified metabolites in different treatments. The PCA of the data showed clear metabolic differences between *F. graminearum*-challenged resistant (Nobeoka) and susceptible (Sonalika) wheat genotypes at 48 hpi as shown in (**Figure 4.4**). In our study, the two principal components explained the 30.32% of the total variance, of which the first principal component (PC1) and the second principal component PC2) attributed 60.6% and 18.5%, respectively. The differences in metabolite responses between the *F. graminearum*-inoculated Nobeoka (resistant) and the Sonalika (susceptible) groups were significant. PCA analysis plot showed that samples fall apart in two different groups based on the variation in metabolite

abundance of the two wheat genotypes inoculated with *F. graminearum* at 48 hpi. The outcomes were then represented graphically in a two-dimensional diagram, where triangles represent data from resistant and the sign, ‘+’ represented susceptible genotypes.

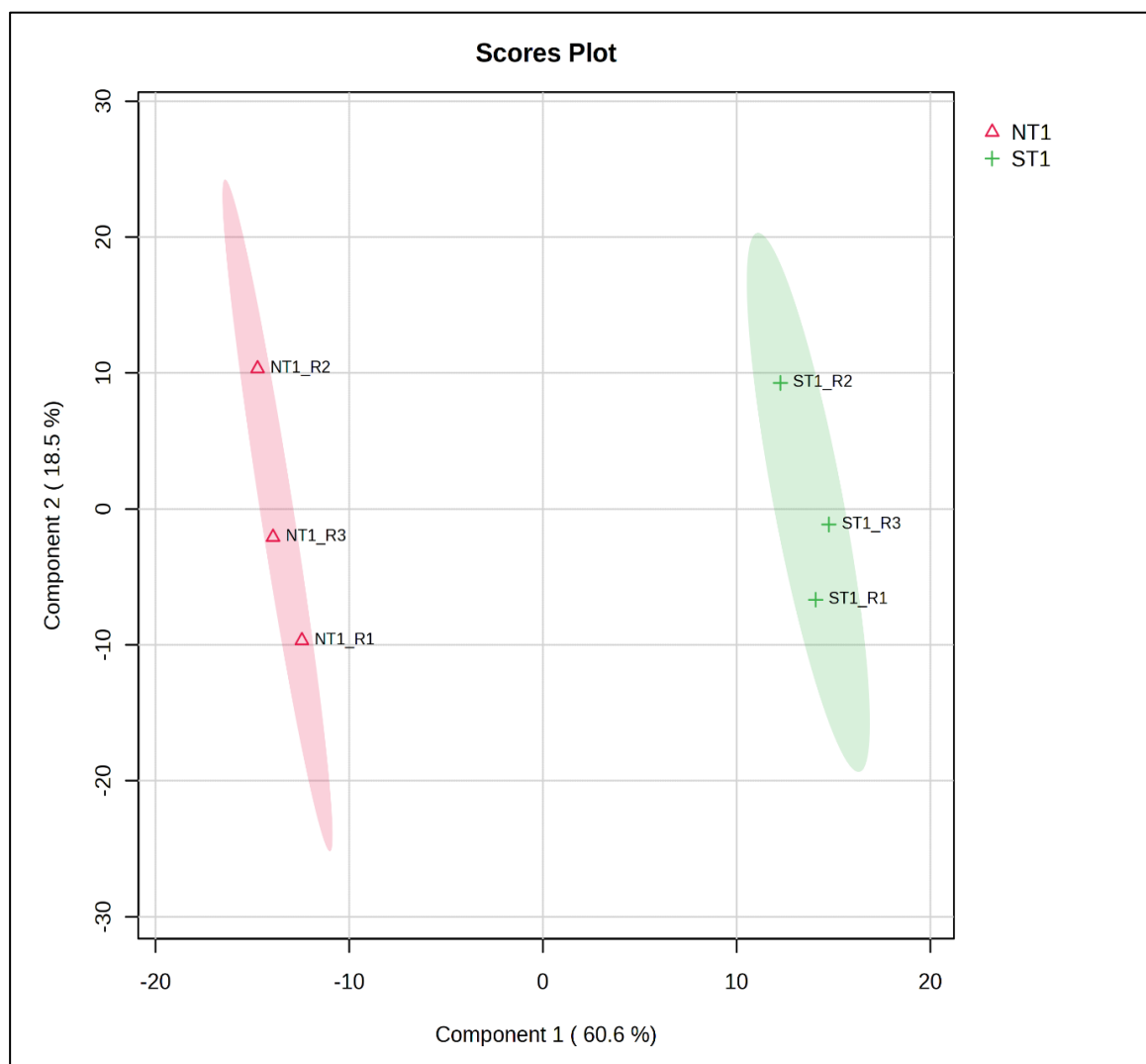


Figure 4.4 Principal component analysis (PCA) score plot of the metabolite profiles of *F. graminearum*-challenged resistant (Nobeoka) and susceptible (Sonalika) genotypes at 48 hpi. Samples were designated as Nobeoka-Treated (NT) and Sonalika-Treated (ST) representing resistant and susceptible sample groups respectively.

4.3.3 Variable Importance in Projection Analysis for Metabolic alterations

The Variable Importance in Projection (VIP) value of the Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) model with threshold > 1 and the fold-change value ≥ 2 or ≤ 0.5 were employed to identify DAMs between the control and *F. graminearum*-challenged resistant and susceptible wheat genotypes (**Supplementary Figure 2**). The VIP score plot presents the top 25 DAMs, including 5,6-dihydroxy-indol-2-carboxylate, 1-naphthol, α -tocopherol, 22- α -hydroxy cholestenol, dTDP-6-deoxy- α -D-allose, selenide, trans-zeatin, felinine, 1-deoxycapsidiol, maltol, β -cryptoxanthine, nitro-benzene, gibberellin 5A and other sugar molecules based on their VIP values shown in (**Figure 4.5 A-B**).

Further, to compare defense responses between resistant (Nobeoka) and susceptible (Sonalika), a pair-wise comparison of VIP scores between NC vs NT and SC vs ST was performed. Comparison between NC vs NT metabolite profiles also showed a significant level of separation of metabolites. The metabolites— feline, α -tocopherol, 2-imminobutanoate, methyl iodide, 1-deoxycapsidiol, C34-phenolphthiotriol, prephenate, nitrobenzene, trans-zeatin, 4-choro catechol, 5-(methyl sulfanyl) (pentanyl-desulphoglucosinolate), tryptamine, Me-JA, 7,9,9-cis-neurosporene, solanidine, and nothofagin were up-regulated in Nobeoka in response to *F. graminearum* suggesting their potential role as resistance-related marker metabolites as visible in (**Figure 4. 5 A**). Conversely. the metabolites— gibberellin A5, 6-hydroxyprotopine, (S)-norcoclaurine, bergapten, and 2-octaprenylephenol were down-regulated in *F. graminearum*-inoculated Nobeoka spikes at 48 hpi suggesting their role in promoting susceptibility to FHB disease in wheat (**Figure 4.5 A**). A distinct separation of metabolites between SC vs ST was observed. The metabolites— methyl ecgonone, dimethylphyloquinone, arsenate, nivalenol, gallate, UDP- α -D-xylose, and vinyl acetate were found to be up-regulated in Sonalika in response to *F. graminearum* indicating their role in facilitating fungal infection (**Figure 4.5 B**).

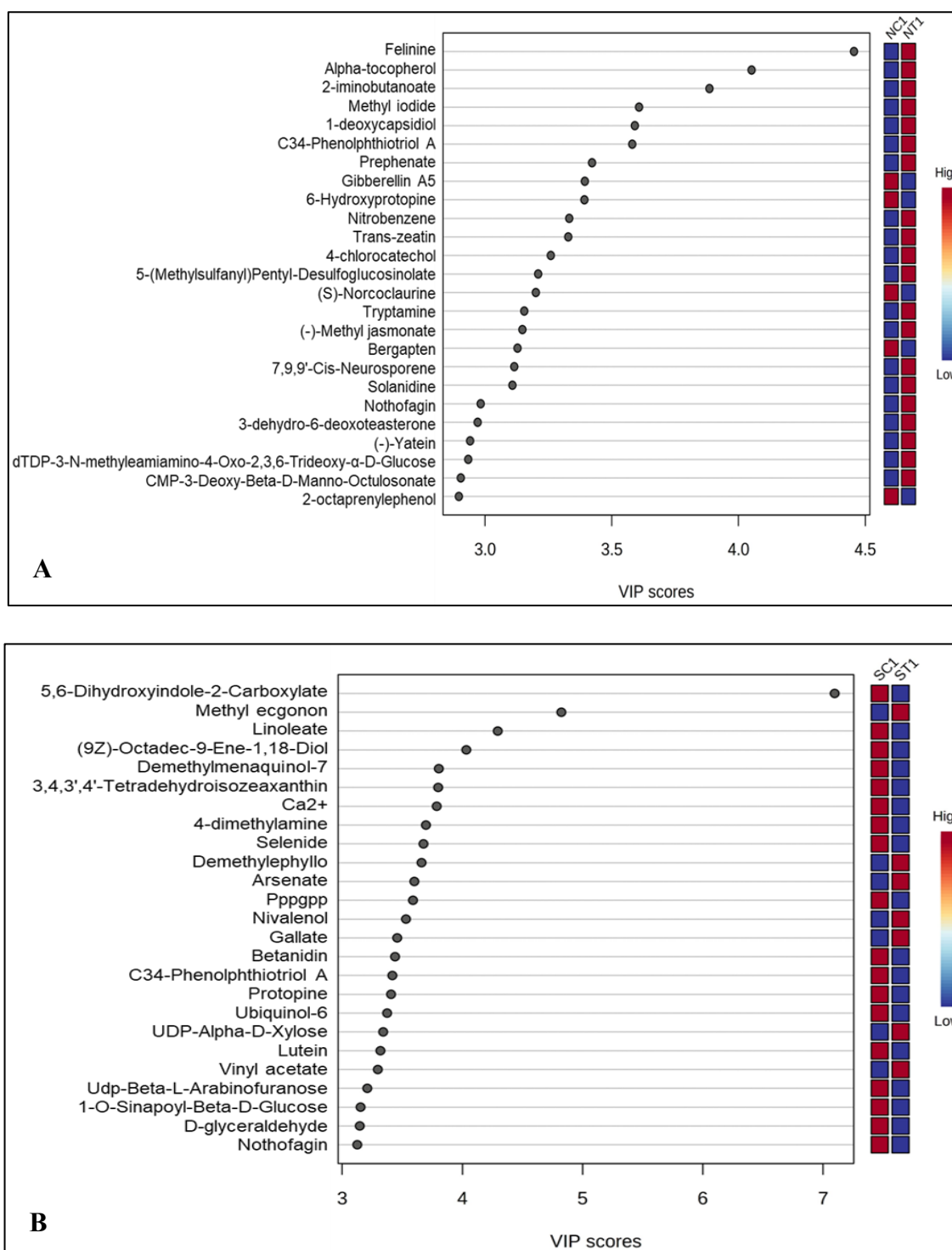


Figure 4.5 Metabolite profiling based on VIP (Variable importance in the projection) scores between *F. graminearum*-challenged (A) Nobeoka (resistant) and (B) Sonalika (susceptible) wheat compared to controls at 48 hpi. VIP scores ≥ 1 were deemed as affected by *F. graminearum* TN01. The colour bar indicates significant levels ranging from highly significant (red color) to less significant (dark blue color).

On the contrary, 5,6-dihydroxy-indol-2-carboxylate, linoleate, dimethyl menaquinol-7, 3,4,3',4'-tetra-dihydro-isoxanthine, calcium⁺², -dimethylamine, selenide, betanidine, C34-phenolphthiotriol A, protopine, ubiquinol 6, lutein, udp-β-L-arabinofuranose, 1-O-senapoyl-β-D-glucose, D-glyceraldehyde, and nothofagin were found to be down-regulated in *F. graminearum*-challenged Sonalika compared to mock-treated samples (**Figure 4.5 B**).

Among the top 30 metabolites that were significantly up-regulated in *F. graminearum*-challenged resistant genotype, Nobeoka compared to control (NC vs NT) at 48 hpi, N-Acetyl-Alpha-D-Glucosamine with a log2 fold-change of 15.46, Ca²⁺ with log2 fold-change of 12.586, α-tocopherol with log2 fold-change of 11 were potential resistant-related metabolite markers (**Table 4.2**). The top 30 metabolites up-regulated in *F. graminearum*-challenged susceptible genotype, Sonalika compared to control (SC vs ST) at 48 hpi also showed high fold-change accumulation.

The metabolite, (9Z)-Octadec-9-Ene-1,18-Diol with a log2 fold-change of 11.44, 2-Dehydro-3-Deoxy-D-Galactonate 6-Phosphate with a log2 fold-change of 11.94, Palmitaldehyde with a log2 fold-change of 10.713 are a few of the metabolites with a potential role in FHB susceptibility (**Table 4. 3**).

Table 4.2 Top 30 metabolites significantly up-regulated in *F. graminearum*-challenged resistant genotype, Nobeoka compared to control (NC vs NT) at 48 hpi

S. No	Metabolite	m/z Ratio	Retention time	log2 (FC)
1	N-Acetyl-Alpha-D-Glucosamine	221.2	6.13	15.46
2	Ca ²⁺	39.8	21.3	12.586
3	Alpha-Tocopherol	430.5	27.1	11
4	Canthaxanthin	564.5	17.8	10.459
5	Carbonate	62.1	7.41	10.72
6	N, N-Dimethylaniline-N-Oxide	137	4.61	10.274
7	Prephenate	225.9	17.05	10.069
8	4-Chlorocatechol	143.85	8.73	9.7962
9	2,3-Diphospho-D-Glycerate	265.95	18.5	9.7232
10	Molybdate	163.71	19.6	9.7131
11	5-(Methylsulfanyl)Pentyl-Glucosinolate	434.9	21.82	9.3496
12	Trans-Zeatin Riboside Monophosphate	430.97	27.29	9.2413
13	(-)-Methyl Jasmonate	224.2	13.5	9.1665
14	Benzyl Alcohol	108.05	24.72	9.0737
15	Trans-Zeatin-9-N-Glucoside	381	27.9	8.9687
16	(+)-Isoitalicene	204.2	10.46	7.8808
17	3-Maleylpyruvate	186	21.34	7.8851
18	Solanidine	397.4	27.49	8.8303
19	(-)-Yatein	400	25.95	8.764
20	Nitrobenzene	125.8	22.69	8.64
21	Glucoiberberin	406.8	29.13	8.614
22	2-Dehydro-3-Deoxy-D-Octonate	237.8	21.26	8.371
23	L-Arginine	174.14	7.82	8.3653
24	(E)-2-Pentenal	84.1	4.45	8.3183
25	18-Oxo-Oleate	296.2	24.98	8.3025
26	Trans-5-O-Caffeoyl-D-Quinate	354	22.45	8.2398
27	D-Alanine	89.12	2.87	8.1833
28	L-Citrulline	175.18	23.24	7.92
29	Pent-1-En-3-One	84.05	8.22	7.8828
30	Delta9-Tetrahydrocannabinol	314.32	21.13	7.8257

Table 4.3 Top 30 metabolites significantly up-regulated in *F. graminearum*-challenged susceptible genotype, Sonalika compared to control (SC vs ST) at 48 hpi

S. No	Metabolite	m/z Ratio	Retention time	log ₂ (FC)
1	(9Z)-Octadec-9-Ene-1,18-Diol	284.17	22.78	11.44
2	2-Dehydro-3-Deoxy-D-Galactonate 6-Phosphate	257.9	20.91	11.094
3	Palmitaldehyde	240.3	23.67	10.713
4	Cyanidin 3-(P-Coumaroyl)-Glucoside	595	20.38	10.642
5	10Beta-Hydroxytaxa-4(20),11-Dien-5Alpha-Yl Acetate	346.4	18.5	10.621
6	7,8-Dihydroneopterin 3'-Triphosphate	494.8	28.91	10.365
7	Demethylphyllquinone	436.3	23.27	10.025
8	L-Ascorbate	176.1	16.03	9.7371
9	Arsenate	141.7	15.02	9.5434
10	Vinyl Acetate	85.9	6.51	9.5335
11	Myristate	228.1	25.6	9.4967
12	C33-Phthiodiolenone A	508.3	24.8	9.4952
13	Red Chlorophyll Catabolite	626.2	26.06	9.3926
14	Nivalenol	312	26.5	9.3059
15	3-(Glutathion-S-Yl) -(3-Methyl) Butanol	393	29.08	9.0204
16	6-Amino-2-Oxohexanoate	145.1	7.02	9.0011
17	Gallate	169.9	8.82	8.9018
18	(Z)-Phenylacetaldehyde Oxime	135.1	9.14	8.7474
19	Pre-Bisucaberin	418.1	27.2	8.6751
20	(Indol-3-Yl) Acetyl-L-Alanine	246	6.3	8.4848
21	(S)-Coclaurine	285	20.1	8.3639
22	(6S)-Hydroxyhyoscyamine	305	21.16	8.2147
23	Cathasterone	432.2	28.59	8.0246
24	(22Alpha)-Hydroxy-Cholesterol	404.2	20.5	7.9893
25	4'-O-Demethylrebeccamycin	554.8	26.98	7.8885
26	(-)-5'-Demethylatein	386	28.7	7.8882
27	Luteolinidin	270.9	28.7	7.8562
28	(-)-Menthol	156.2	16.35	7.7704
29	Acibenzolar-S-Methyl	209.9	25.91	7.7454
30	3-Methylcatechol	123.9	24.6	7.6834

Table 4.4 Top 30 metabolites significantly up-regulated in *F. graminearum*-challenged wheat spikes between the treatments, Nobeoka Treated (NT) vs Sonalika Treated (ST) at 48 hpi

S. No	Metabolite	m/z Ratio	Retention time	log2 (FC)
1	Selenide	81.8	4.97	12.197
2	Betaine Aldehyde Hydrate	121	3.47	10.483
3	Coproporphyrinogen Iii	660.2	8.51	10.227
4	3,4,3',4'-Tetradehydroisoeaxanthin	564.4	25.2	10.222
5	4-Dimethylaminophenylazobenzene	225.1	5	10.216
6	Dtdp-4-Dehydro-Beta-L-Rhamnose	545.9	22	9.7913
7	N, N-Dimethylaniline-N-Oxide	137	4.61	9.7685
8	Gibberellin A51	332.1	21.8	9.7484
9	Gdp-Beta-L-Galactose	604.8	29.2	9.6224
10	Methylglyoxal	71.9	2.16	9.2678
11	Molybdate	163.7	19.6	9.2408
12	(-)-Methyl Jasmonate	224.2	14.9	9.2383
13	2-Hydroxy-2-Methylpropanenitrile	84.8	2.12	8.9207
14	Dudp	387.8	29.4	8.8066
15	Trans-Zeatin-9-N-Glucoside	381	27.9	8.4962
16	Cis-Stilbene Oxide	196	22.4	8.3579
17	(9Z)-12,13-Dihydroxyoctadeca-9-Enoate	314.2	28.6	8.2621
18	Betanidin	390	27.2	8.0201
19	L-Arginine	174.1	7.82	7.8599
20	D-Alanine	89.1	2.87	7.678
21	Deoxypodophyllotoxin	398.1	28.15	7.4355
22	Pent-1-En-3-One	84.05	8.22	7.4101
23	(+)-Isoitalicene	204.2	10.46	7.3756
24	Delta9-Tetrahydrocannabinol	314.3	21.13	7.3205
25	Solanidine	397.3	28.5	7.2074
26	Dimboa-Beta-D-Glucoside	373	28.08	6.9556
27	3-Phospho-D-Glyceroyl-Phosphate	265.9	27.84	6.8901
28	10Beta-Hydroxytaxa-4(20),11-Dien-5Alpha-Yl Acetate	340.7	10.25	6.7678
29	3-Beta-Hydroxyandrost-5-En-17-One 3-Sulfate	368.1	19.36	6.6249
30	Spermidine	145.2	14.55	6.3096

4.3.4 Volcano plot analysis

In agreement with the VIP plot analysis, the volcano plot also revealed key metabolites with significant log fold-change accumulation in response to the fungal treatment. A distinct metabolite profile was revealed in the volcano plot depicting NC vs. NT in comparison to SC vs. ST as observed in **(Figure 4.6)**. The metabolites— 4, chloro catechol, glutathione, glucoiberverin, Me-JA, E-2-pentenal, yatein, prephenate, solanidine, α -tocopherol, and benzyl alcohol were up-regulated in Nobeoka in response to *F. graminearum* indicating their role in FHB resistance in wheat. Whereas the downregulated metabolites were 5-amino-1-(5-phospho- β -D-Ribosyl) imidazole, 5-methyl-L-methionine, chorismate, palmitoleate, 8-(methyl sulfanyl) octyl-desulfoglucosinolate, (22 α)-hydroxyl-cholestanol, dihydro-sanguinanine and 6-hydroxy-protopine in those samples at 48 hpi shown in **(Figure 4. 6)**.

In *F. graminearum*-challenged Sonalika, the up-regulated metabolites were palmitaldehyde, propenylsulfenate, dimethylphyloquinone, C33- phenolphthiotriol, vinyl acetate, arsenate, gallate and nivelanol while the down-regulated metabolites were Ca^{+2} , 6-dihydroxy-indol-2-carboxylate, selenide, Cdp-glycerol and 3,4,3',4'-tetra-dihydro-isoxanthine indicating them as targets for the fungal invasion and may serve as susceptibility-related metabolite markers **(Figure 4.7)**.

To identify the metabolites altered in response to *F. graminearum* in both Sonalika and Nobeoka, the top 30 metabolites significantly up-regulated in *F. graminearum*-challenged wheat spikes between the treatments, Nobeoka Treated (ST) vs Sonalika Treated (NT) at 48 hpi were analyzed. The volcano plot of NT vs ST samples showed up-regulation of the metabolites, selenide, coproporphyrinogen, Me-JA, L-alanine, 3,4,3',4'-tetra-dihydro-isoxanthine and solanidine as shown in **(Figure 4.7)**. In agreement, these compounds were also present in the top 30 listed compounds detected in NT vs ST samples with a fold-change accumulation for selenide with log2

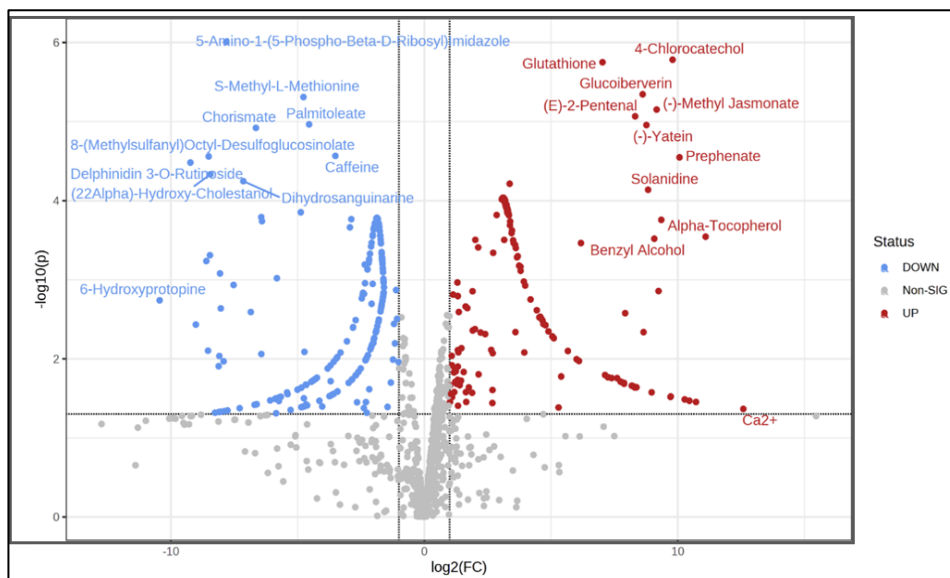


Figure 4.6 Volcano plots showing up-regulated and down-regulated metabolites in NC vs NT. Metabolites with a fold change greater than 2 (x-axis) and a t-test p-value less than 0.05 (y-axis) were marked with red circles. Both fold changes and p-values are log-transformed.

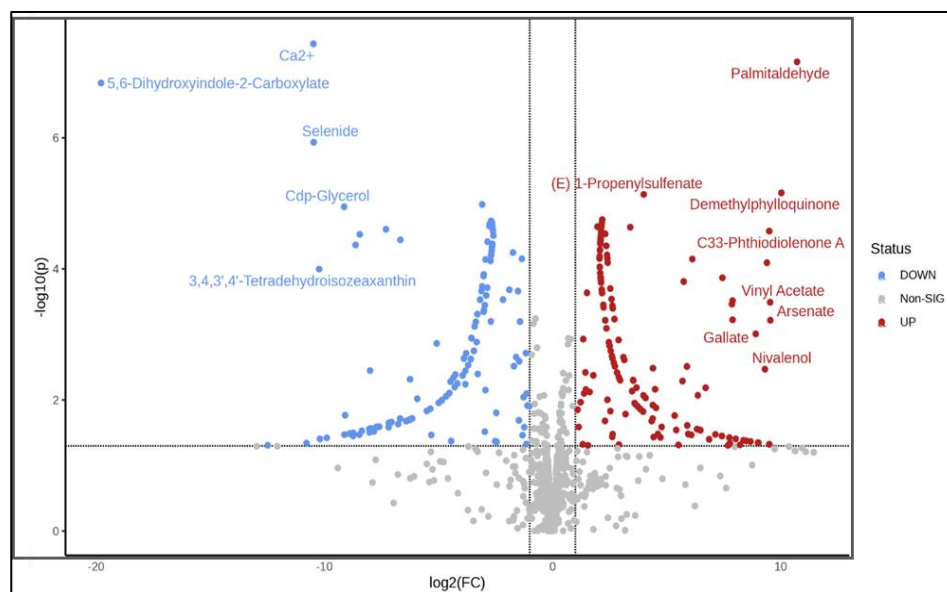


Figure 4.7 Volcano plots showing up-regulated and down-regulated metabolites in SC vs ST samples. Metabolites with a fold change greater than 2 (x-axis) and a t-test p-value less than 0.05 (y-axis) were marked with red circles. Both fold changes and p-values are log-transformed. The distance from the origin (0,0) corresponds to the metabolite's significance, with greater distances indicating higher significance.

fold-change of 12.197 followed by betaine aldehyde hydrate with log2 fold-change of 10.483 and coproporphyrinogen Iii with log2 fold-change of 10.227 (**Table 4.4**). These up-regulated metabolites could serve as resistance-related (RR) metabolite markers. Whereas, the down-regulated metabolites that could serve as susceptibility-related (SR) metabolite markers are β -cryptoxanthin, chorismate, dTDP-6-deoxy- α -D-allose, 9,10-epoxystearate, (R)-prunasin, delphinidine-3-O-rubinoside, cis-dienelactone, luteolinidine, hydroxyprotopine, and gibberellin A5 as shown in (**Figure 4.8**).

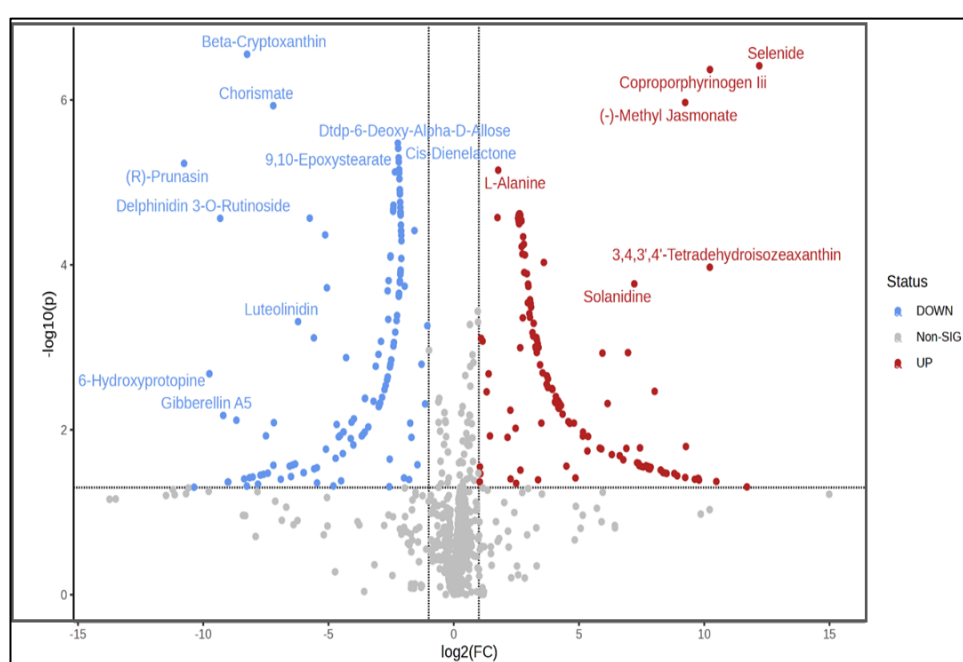


Figure 4.8 Volcano plot showing up-regulated and down-regulated metabolites in ST vs. NT samples. Metabolites with a fold change greater than 2 (x-axis) and a t-test p-value less than 0.05 (y-axis) were marked with red circles. Both fold changes and p-values are log-transformed. The distance from the origin (0,0) corresponds to the metabolite's significance, with greater distances indicating higher significance.

4.3.5. Corelation Heat Map Analysis

A correlation heat map was plotted for the top 50 significant metabolites to analyze their role in host-pathogen interaction as shown in (**Figure 4.9**). The characteristics of the potential metabolites

differently generated across the two sample groups were identified. The differential metabolites illustrated in the heatmap demonstrated distinct expression patterns between resistant and susceptible genotypes as well as their responses to *F. graminearum* infection. Among those metabolites, Me-JA, alanine, selenide, coproporphyrinogen, 5- α -cholestanol, cis-zeatin-riboside, lutein, UDP- β -L-arabinose, D-proline, C33-phyllodiolenon, nothofagin, and E-1-glutathione were some of the significantly accumulated metabolites in *F. graminearum*-challenged Nobeoka (NT) samples at 48 hpi indicating their role in resistance. The compound, Me-JA was found to be prominent in Nobeoka-treated with *F. graminearum* (NT) samples at 48 hpi indicating its role in resistance against *F. graminearum* at later stages of infection. Whereas metabolites like delphinidin-3-O-rutinoside, adenylosuccinate, β -cryptoxanthin, (R)-prunasin, 1-naphthol, D-lysine, SA, gibberllinA20, 6-hydroxyprotopine, luteolinidin, β -cryptoxanthin, dihydroxyacetone, 2-Octa-prenylphenol, UDP- α -D-xylose, capsidiol, cis-dienelactone, chorismate, and 9, 10-eoxystearate were up-regulated in Sonalika in response to *F. graminearum*.

Further, the altered metabolites detected through the PLS-DA model in response to *F. graminearum* were subjected to hierarchical cluster analysis (HCA) to validate outcomes from the PCA and PLS-DA analyses. The HCA model which utilizes the Euclidean distance and complete linkage grouping to detect the clustering patterns among the metabolites, revealed a similar outcome as obtained by PCA and PLS-DA models, confirming the validity of all the multivariate models used in this study (**Supplementary Figure 3**). The findings confirmed the downregulation of the metabolites in defense such as coproporphyrinogen, dTDP-4-dehydro- β -L-rhamnose, lutein, ubiquinol 6, UDP- β -L-arabinofuranose in *F. graminearum*- challenged Sonalika (ST) when compared to in *F. graminearum*- challenged Nobeoka (NT) samples.

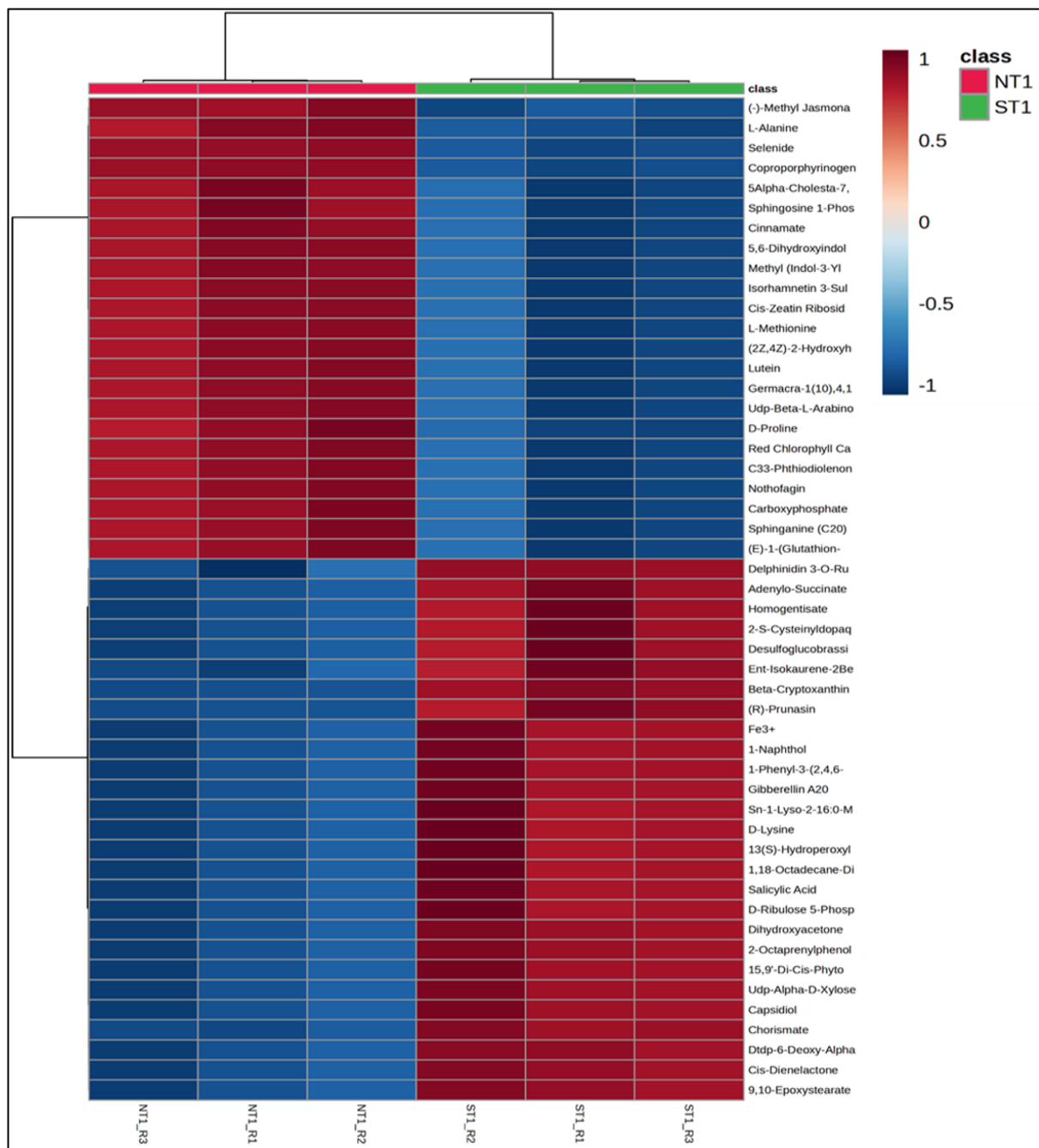


Figure 4.9 Correlation heat map of top 50 metabolites detected in spikes of FHB susceptible, Sonalika (ST) and FHB resistant, Nobeoka (NT) challenged with *F. graminearum* TN01 at 48 hpi. Differentially expressed metabolites were indicated in bold color. The x- and y- axes represent the significance levels related to metabolic pathways. The color scale from blue to red represents differentially abundant metabolites (DAMs) in proportion to the metabolic pathway.

4.3.6 KEGG pathway analysis

The KEGG pathway enrichment also showed beta-alanine, nicotinate, and nicotinamide metabolism, sulfur, and nitrogen metabolism in our study at 48 hpi in *F. graminearum* inoculated wheat genotypes with the highest rich factor value. The altered pathways were revealed by enrichment and topology analysis of the top 25 metabolites. The x-axis represents pathway enrichment, whereas the y-axis represents the pathways affected by *F. graminearum* challenge in wheat. Large sizes and dark colors represent major pathway enrichment and high pathway impact values. The pathway enrichment analysis showed high intensity of the metabolites pertaining to glycine, serine, and threonine metabolism followed by arginine biosynthesis and valine, leucine, and isoleucine biosynthesis. The other pathways that were highly enriched were pyrimidine metabolism, glyoxylate and dicarboxylate metabolism, cysteine and methionine metabolism, alanine, aspartate and glutamate metabolism, glycolysis and gluconeogenesis, pantothenate and CoA biosynthesis, citrate cycle (TCA-Cycle), puruvate and, arginine and proline metabolism as shown by a bar graph (Supplementary Figure 4 A) and a dot plot (Supplementary Figure 4 B).

4.3.7 Defense and Mycotoxin Gene Expression Analysis

To gain a deeper insight into the wheat metabolome associated with defense and mycotoxin synthesis, the expression of two defense genes—*PR1* and *PDF1.2* representing SA and JA mediated-defense signaling respectively and two Trichothecene synthesis genes—*TRI5* and *TRI6* were compared between *F. graminearum*-challenged Nobeoka and Sonalika in relation to controls in a time course study at 24, 48 and 72 hpi. The results revealed significant and differential expression of those genes revealing variation between the two wheat genotypes and the time points of study analyzed post-Fusarium inoculation as shown in (Figure 4. 10). An up-regulation of the *PR1* gene was observed in Nobeoka at all time points compared to Sonalika in response to *F. graminearum*. The mRNA transcripts of *PR1* gene accumulated with an increased fold-change (>1.5) in *F.*

graminearum- challenged Nobeoka at 24 and 48 hpi which gradually decreased at 72 hpi indicating the activation of SA-mediated defenses at early stages of infection (**Figure 4.10 A**).

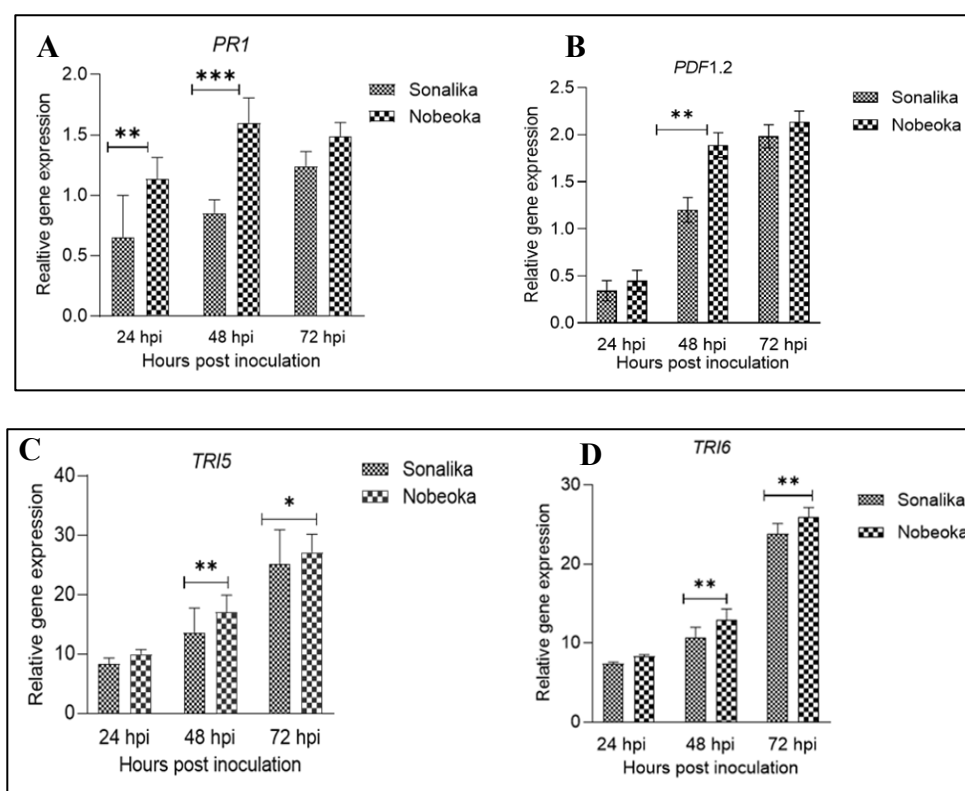


Figure 4.10 Relative gene expression studies by Real-time quantitative qRT-PCR. (**A-B**) Defense and (**C-D**) Mycotoxin gene expression in a time-course study at 24, 48, and 72 hpi. The defense genes: *PR1* (**A**), *PDF1.2* (**B**), and mycotoxin genes: *TRI5* (**C**), and *TRI6* (**D**) were analyzed in Nobeoka (resistant) and Sonalika (susceptible) genotypes infected by *F. graminearum* TN01. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method. Fungal-specific (β -*Tubulin*) gene was used as an internal control. Non-template DNA control (NTC) was used as a negative control. The real-time data was plotted using SigmaPlot 11.0 and the statistical significance was tested by ANOVA at different time points. The significance level of variation between samples is indicated as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Whereas the expression of *PDF1.2* (**Figure 4.10 B**) was relatively significant at later time-points of inoculation (48 and 72 hpi) in both the genotypes in response to *F. graminearum*. The *PDF1.2* gene expression level was at low levels at 24 hpi in both the genotypes which increased to >2.0-fold change in Nobeoka at 48 and 72 hpi in response to *F. graminearum*. Similar upregulation was

observed at 72 hpi in Sonalika in comparison to control samples indicating the activation of JA-mediated defenses at later stages of infection. The expression of mycotoxin genes *TRI5* (Figure 4.10 C) and *TRI6* (Figure 4.10 D) transcripts was significant at all time points in both the genotypes. The level of expression was higher in the resistant genotype, Nobeoka compared to the susceptible genotype, Sonalika suggesting their function in virulence to activate resistance (R) gene-mediated defense in wheat.

4.3.8 Effect of exogenous DON

To analyze the effect of the mycotoxin, DON on *F. graminearum*, the mycelial growth of the fungal pathogen was monitored on PDA plates containing different concentrations of DON (1 ppm to 3 ppm) along with the control without DON treatment.

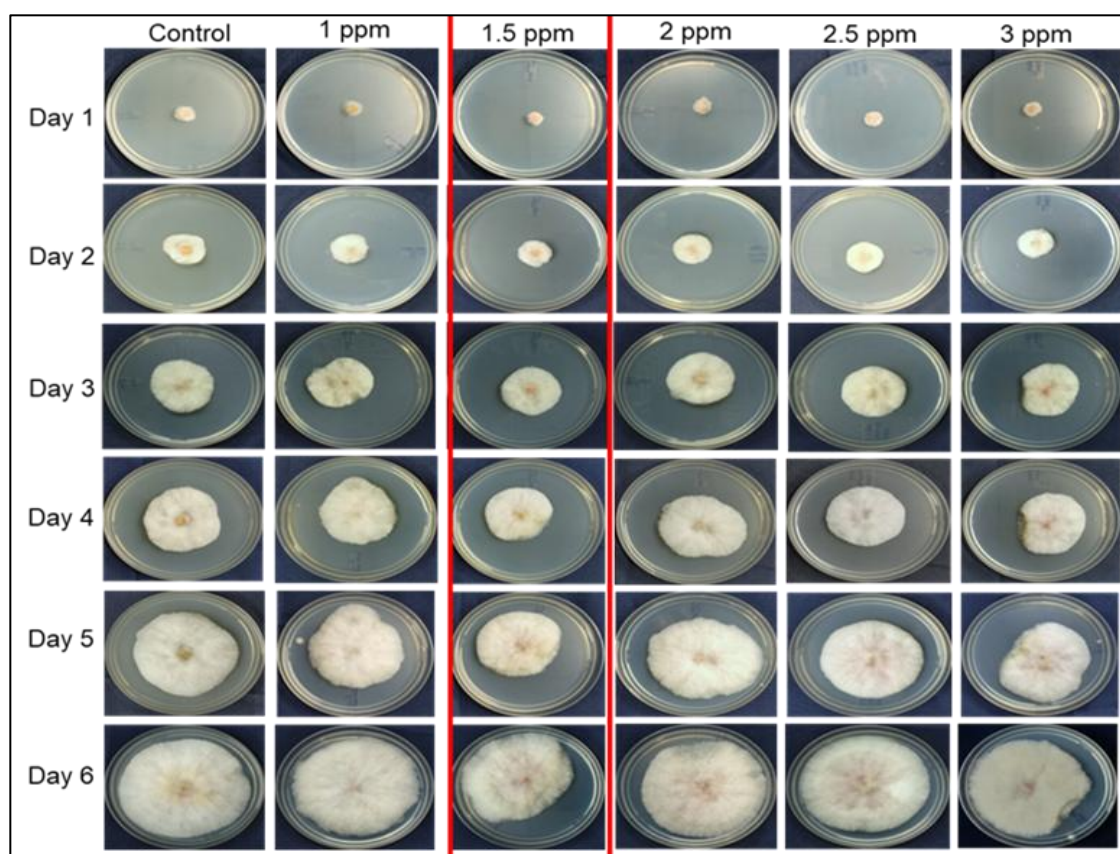


Figure 4.11 Effect of exogenous DON treatment on the colony morphology of *F. graminearum*.

In comparison to the control, the DON-treated fungus did not show any significant changes in both colony morphology or radial growth inhibition at all concentrations except at 2.5 ppm concentration showed minimal variation at all concentrations, except at 1.5 ppm with a marginal change in both colony size (**Figure 4.11**) and radial growth inhibition is shown graphically in (**Supplementary Figure 5**).

4.4 DISCUSSION

The host-pathogen interactions involving the two wheat genotypes, resistant (Nobeoka) and susceptible (Sonalika), with *F. graminearum*, served as incompatible and compatible interactions were carried out to detect and analyse the distinct metabolites with a role in resistance and susceptibility, respectively. The metabolomics study was carried out at the time point of 48 hours post-challenge with the pathogen, since this time point is critical for the hemibiotrophic fungus, such as *F. graminearum*, which initially leads a biotrophic phase and switches to a necrotrophic phase at a later stage of infection in the host (Kheiri et al., 2019). Previous studies have shown that the mechanisms underlying plant-pathogen interactions, as well as the defense-related compounds, could be detected from plant metabolomes challenged with fungal pathogens. Metabolomics has been successfully used to detect FHB resistance biomarker metabolites in barley against *F. graminearum* (Kumaraswamy et al., 2011). Five FHB-resistant and FHB-susceptible barley genotypes were analyzed to identify resistance and susceptibility mechanisms in barley against the fungal pathogen, *F. graminearum*. Kumar et al. (2015) identified the altered disease resistance pathways in chickpea–fusarium interaction. Likewise, *Fusarium oxysporum*-induced metabolic reprogramming was detected in the roots of chickpeas challenged with the fungal pathogen (Kumar et al., 2016). A GC-MS based targeted metabolic profiling led to the detection of changes in wheat in response to the chemical treatment with DON (Warth et al., 2015).

In our study utilizing GC-MS, a total of 809 metabolites representing various groups of compounds were detected using both univariate and multivariate analyses. Likewise, in earlier studies using the LC-LTQ-Orbitrap detection method, 340 metabolites were detected based on wheat near-isogenic lines (Gunnaiah et al., 2012). Previously, using GC-MS, 41 resistance-related (RR) metabolites were identified by univariate analysis of variance, and 45 resistance

function-associated metabolites were detected by multivariate analysis (Hamzehzarghani et al., 2008). In one study conducted by Sunic et al. (2023), it was confirmed that ten metabolites were identified as potential resistance-related compounds in the grains of six FHB-resistant varieties.

A correlation heat map revealed Me-JA, alanine, selenide, coproporphyrinogen, 5- α -cholestanol, cis-zeatin-riboside, lutein, UDP- β -L-arabinose, D-proline, C33-phyllodiolenon, nothofagin and E-1-glutathione, as some of the significantly accumulated metabolites in *F. graminearum*-challenged Nobeoka (NT) samples at 48 hpi indicating their potential role as resistance-related metabolite markers. On the contrary, metabolites like delphinidin-3-O-rutinoside, adenylosuccinate, β -cryptoxanthin, (R)-prunasin, 1-naphthol, D-lysine, SA, gibberellinA20, 6-hydroxyprotopine, luteolinidin, β -cryptoxanthin, dihydroxyacetone, 2-Octaprenylphenol, UDP- α -D-xylose, capsidiol, cis-dienelactone, chorismate and 9,10-eoxystearate were up-regulated in Sonalika in response to *F. graminearum* suggesting them as targets of *F. graminearum*. Likewise, the metabolites– methyl ecgonone, dimethylphyloquinone, arsenate, nivalenol, gallate, UDP- α -D-xylose and vinyl acetate were found to be up-regulated in Sonalika in response to *F. graminearum*.

Specific metabolites were differentially accumulated in response to *F. graminearum*. The level of amino acids, flavonoids, organic acids, inorganic compounds, terpenoids, fatty acid derivatives, and other related phytochemicals, were markedly elevated in Nobeoka when compared to Sonalika. Amino acids including proline and alanine were shown to accumulate during FHB resistance (Zhao et al., 2021). Proline provides resistance to both abiotic and microbial plant diseases, with resistant plants showing higher proline levels compared to susceptible plants (Fabro et al., 2004; Cecchini et al., 2011). Flavonoids are low-molecular-weight secondary metabolites that are abundant in plants and have several functions, including signaling between plants and microorganisms (Groenbaek et al., 2019) and biotic stress defense

(Sukumaran et al., 2018). The bulk of the flavonoids accumulated mostly in the spikes of *F. graminearum*-inoculated Nobeoka. Also, antioxidants such as alpha-tocopherol, lipoic acid, and L-ascorbate were differentially accumulated in wheat in response to *F. graminearum*. Overall, metabolites of this category indicated a higher antioxidant, and ROS scavenging activity during *F. graminearum* infection. Flavonoids and other antioxidants such as alpha-tocopherol help to scavenge ROS generated during infection, thus protecting plant cells from oxidative damage. Additionally, certain metabolites can bind to mycotoxins, reducing their toxicity and facilitating their removal from the plant system.

The several other metabolites that were up-regulated in Nobeoka compared to Sonalika include xanthoxin, solanidine, Me-JA, chlorocatechol, alanine, cinnamate, methionine, selenide, and glutathione. Xanthoxin a precursor of abscisic acid (ABA) is involved in the plant defense and some of the fungi causing the powdery mildew were also known to produce ABA with a potential role in the defense, growth, and development of the plant. Similarly, trans zeatin one of the isomers of zeatin (cytokinins) has been shown to play a role in plant immunity. The increased levels of trans zeatin in our study are in line with the study previously carried out by (Grobkinsky et al., 2013) where they showed that the elevated levels of trans zeatin actively promote resistance specifically against hemibiotrophic pathogens such as *Pseudomonas syringae*. The metabolite, *Demethylmenaquinol-7* was identified as differentially abundant in our study. In addition to its role as a fungal effector molecule, it inhibits the salicylic acid (SA)-mediated plant defense response, resulting in the down-regulation of the *PR1* gene. This interference with the ethylene (ET) signaling pathway facilitates fungal colonization and growth within host plants.

In our analysis, several metabolites like benzoquinone, benzyl alcohol, 2,5, dihydroxypyridine, pyroline carboxylate, bisucaberin, and glucosinolates (Consonni et al., 2010) have been identified as having antifungal, signaling, and cell wall reinforcement properties. Several

metabolites can serve as fungistatic agents to inhibit the mycelial growth of fungal pathogens. Compounds like phenylalanine, spermidine, tryptophan, maleic acid, fumarate, fructose, nicotinamide, and cinnamic acid were upregulated in these genotypes, likely functioning to resist pathogen infection (Pang et al., 2021). Increases in p-coumaric acid and sinapylaldehyde, precursors of syringyl-lignin (S-type), have been reported as potential FHB-resistance biomarkers during wheat head infection (Bollina et al., 2010, 2011; Gunnaiah & Kushalappa, 2014).

The resistant genotype (Nobeoka) was associated with higher amounts of various fatty acids, primarily stearic and palmitic acids, aromatic compounds such as p- and m-coumaric acids, and sugars like myoinositol. Metabolites with significantly ($P < 0.05$) higher abundance in the resistant genotype compared to the susceptible were classified as resistance-related (RR) metabolites. Conversely, significantly lower abundances of these metabolites were regarded as susceptible (Surendra & Cuperlovic-Culf, 2017). Most of these metabolites identified in our study might possess a potential role as RR metabolites.

A significant number of secondary metabolites produced by plants are critical for direct plant defense. Among these metabolites are gramine, linoleic acid, phenylalanine, carotenoids, phenolic acids, cinnamic acids, and benzoxazinoids which were differentially accumulated. Benzoxazinoids, also known as Bx metabolites, such as DIMBOA (4.68-fold), and Dimboa-b-glucosinolate emerge as important contributors to resistance in our study and were in line with the study carried out by Murphy & Zerbe (2020). In the case of wheat, *T. aestivum*, these compounds include benzoxazinoids (Bx) and phenolic acids. Research studies showed that the levels of benzoxazinoids vary among different wheat cultivars (Maria et al., 2008). Benzoxazinoids also function as defense compounds against fungal pathogens (De Bruijn et al., 2018). *Fusarium* species possess a notable ability to detoxify Bx metabolites. This accumulation has been linked to resistance against FHB in wheat and wild barley heads. In

wheat, the accumulation of benzoxazinoids is regulated by JA (Sue et al., 2021). Notably, the accumulation of benzoxazinoids is positively correlated with resistance to disease and insects in maize, wheat, and rye (Ahmad et al., 2011; Meihls et al., 2013). It will be interesting to investigate the potential of benzoxazinoid (Bx) metabolites in enhancing resistance in wheat to FHB caused by *F. graminearum*.

One of the metabolites, coproporphyrinogen plays a role in the plant defense mechanism, fungal interaction, signaling pathways, growth, and virulence. We have observed that coproporphyrinogen is an abundantly present metabolite in our study and might be involved in reducing and detoxifying the mycotoxins in resistant genotype Nobeoka. Similarly, in plant-fungal interactions, artemisinate has been shown to significantly suppress fungal growth, disrupt hyphae formation, and even influence gene expression in ectomycorrhizal fungi and phytopathogenic *Fusarium*. In our analysis, we observed that artemisinate was differentially abundant in both the *Fusarium*-challenged wheat genotypes.

The production of defense-related metabolites in resistant wheat genotypes has increased, impeding pathogen progression through various mechanisms. Phenolic compounds, for instance, directly inhibit fungal growth by disrupting cell wall integrity and enzyme activity. Studies conducted by Al-Khayri et al, (2023) also demonstrated that the amounts of phenolic acids can vary among different varieties of wheat (Zhang et al., 2018). Likewise, in our study, Nobeoka (resistant to FHB) displays higher levels of phenolic compounds. This is consistent with the idea that these compounds play a significant role in the plant's defense against the pathogen during the interaction between the wheat plant and the *Fusarium* fungus.

The compound Me-JA was found to be prominent in Nobeoka-treated with *F. graminearum* (NT) samples at 48 hpi indicating its role in resistance against *F. graminearum* at later stages of infection. On the contrary, SA and chorismate, related to the SA pathway, were up-regulated

at 48 hpi, signifying the antagonistic action of both SA and JA-mediated pathways. Earlier studies also demonstrated that plant hormones such as JA facilitates *F. graminearum* spread in the early stages of infection in its host (Fan et al., 2019; Hou & Tsuda., 2022), whereas at later stages it promotes resistance in the wheat plants (Nalam et al., 2015). Pentacyclic oxylipins such as Me-JA play a decisive role in plant defense against necrotrophic fungi (Blée, 2002). These oxylipins are mostly used as signaling molecules to influence fungal growth, virulence, and defense mechanisms. The biosynthesis of Me-JA through the octadecanoid pathway starts with linolenic acid, a metabolite that is enriched in *F. graminearum*-inoculated samples. Our metabolomic analysis revealed a significantly enriched KEGG pathway for plant hormone signal transduction. The JA derivative, Me-JA was upregulated 9.10-fold, while its bioactive conjugate (jasmonoyl-L-isoleucine) was upregulated 3.55-fold at 48 hours post-infection. Our study therefore shows the abundance of oxylipins in the *Fusarium*-inoculated wheat genotypes such as 16-oxo-palmitate (8.78-fold), 3-oxo-palmitate (8.23-fold), and 18-oxo-oleate (8.25-fold) which is in agreement with the previous findings (Blée, 2002).

Recent years have witnessed great progress in the elucidation of the biosynthesis of these monocot terpenoid phytoalexins (Block et al., 2019). While sesquiterpenoids are typically derived from cytosolic isoprenyl precursors via the mevalonate pathway, diterpenoids generally originate from the plastidial precursors provided by the methylerythritol phosphate (MEP) pathway (Liu et al., 2024). In our study, deoxycapsidiol one of the sesquiterpenoid compounds, acts as the precursor for Capsidiol having phytoalexin properties involved in the plant defense. This compound has been associated with playing a role in the plant's defense against fungal pathogens. Mandujano et al. (2000) in one of their studies have shown that fungal elicitors like Me-JA influence the sesquiterpenoid biosynthesis pathway and the related enzymes such as deoxycapsidiol and sesquiterpene cyclase. Therefore, this supports our findings that deoxycapsidiol was a differentially abundant metabolite present in our study as well.

Deoxycapsidiol has been shown to act as a virulence factor for *Pseudomonas syringae* by inhibiting the JA-mediated plant defense, thereby impacting the plant's ability to resist fungal growth.

In response to *F. graminearum*, the host plants engage diverse defensive mechanisms, such as DON detoxification, alterations in host metabolism, adjustments to cell wall development, and the synthesis of antimicrobial compounds and *PATHOGENESIS RELATED (PR)* proteins (Jia et al., 2009; Pasquet et al., 2014). The *PR1* and *PDF1.2*, which were shown to be involved in SA and JA pathways, respectively, showed upregulation in both susceptible and resistant genotypes in comparison to the water-inoculated control.

Plant hormones like gibberellins (GAs) and auxin are known to be vital for plant development, reproduction, metabolic processes, and adaptation to various environmental challenges. The phytohormone Gibberellin varies significantly between resistant and susceptible wheat genotypes. Our study revealed several gibberellin-related metabolites, including gibberellin A51, gibberellin A4, gibberellin A5, gibberellin A9, gibberellin A17, gibberellin A12, gibberellin A20, gibberellin A37, that were differentially accumulated upon FHB inoculation. Studies have revealed that *GA51* promotes the phenylalanine ammonia-lyase activity one of the crucial enzymes responsible for isoflavone synthesis, and isoflavones have been shown as an important part of the plant defense system against fungal pathogens (Barnes and Jones, 1984).

Previous studies by Desmond et al. (2008), have demonstrated that DON accumulation plays an important role in *F. graminearum* virulence and colonization in host tissues. Our findings from the wheat metabolome analysis in response to *F. graminearum* at 48 hpi showed an increase in the concentration of mycotoxins, such as DON in the spikes of both the wheat genotypes. Further, the gene expression studies also revealed up-regulation of mycotoxin-specific genes, *TRI5* and *TRI6* in spikes of both Nobeoka and Sonalika. A similar increase in

the DON level was also noted in both the susceptible cultivar, Hanseat, and the resistant cultivar, Arina previously by Waśkiewicz et al. (2014). This increase in DON level is reported to be associated with a switch to the necrotrophy state of the fungus (Gunupuru et al., 2017).

Earlier studies have shown that resistant wheat cultivars can degrade DON into less toxic compounds, thereby mitigating its harmful effects (Kazan et al., 2012). Our findings also showed lower levels of DON in Nobeoka suggesting that these genotypes possess mechanisms to either inhibit mycotoxin production or more effectively detoxify these compounds. Other trichothecene mycotoxins that are commonly produced by *Fusarium* fungi such as Nivalenol (9.22-fold) a highly abundant metabolite observed in the Sonalika treated samples in comparison to the resistant genotype Nobeoka, Fusarenone X (4.09-fold) a member of type B trichothecenes (Aupanun et al., 2017), Triketide pyrone (4.47-fold), and T-2 Toxin (2.62) were present in susceptible genotype with significant fold change. Thus, it can be asserted that, during the infection process, the mycotoxins are produced as an intricate tactic by the fungal pathogen to bypass and exploit the host defense systems (Diamond et al., 2013). Additionally, Triketide pyrone and tetraketide pyrone are found to be important secondary metabolites with phytotoxin and phytoalexin properties and also act as the signaling molecule by impacting the plant defense, growth and susceptibility to fungal pathogens, thereby acting as a virulence factor. Furthermore, the production of the mycotoxin DON and the generation of superoxide anion ($O_2^{\bullet-}$) in the resistant cultivar during the late phase post-inoculation were reduced relative to the susceptible cultivar. The DON accumulation in *F. graminearum*-inoculated spikes has been found to stimulate the production of H_2O_2 by expanding the hyphal spread, which subsequently triggers PCD in wheat and facilitates its transition from biotrophy to necrotrophy (Bönnighausen et al., 2019). Studies conducted to analyze the effect of the DON on the viability of Arabidopsis cells showed that DON inhibited apoptosis-like programmed cell death (PCD) in the treated host cells (Diamond et al., 2013). Likewise, to check if DON

has any effect on colony growth and radial growth inhibition, we treated *F. graminearum* with different concentrations of DON and observed a marginal effect on the fungal growth as shown in (Supplementary Figure 5).

Further, a diverse set of metabolites potentially suppressing FHB symptoms were identified in wheat. The HCA model along with PCA and PLS-DA models confirmed the down-regulation of the metabolites in defense such as coproporphyrinogen, dTDP-4-dehydro- β -L-rhamnose, lutein, ubiquinol 6, UDP- β -L-arabinofuranose in *F. graminearum*-challenged Sonalika when compared to NT sample suggesting their role in enhancing FHB susceptibility in Sonalika. Among the metabolites, there are bioactive compounds, quinones, coumarins, tannins, vitamins, and terpenoids. Acteoside, isoacteoside, verbascoside, and plant amajoside are some of the phenolic acids predominantly found in the infected spikes of both genotypes. All of the precursor molecules linked to lignin biosynthesis—p-coumaraldehyde, p-coumaryl alcohol, caffeic acid, coniferyl alcohol, ferulic acid, and coniferin (Kumar et al., 2020) increased during FHB infection. Lignans are predominantly observed in resistant genotypes, which are involved in host defenses against fungal pathogens. FHB resistance in wheat is reportedly linked to the phenylpropanoid, terpenoid, and fatty acid pathways that play a role in plant defense signaling, antimicrobial activity, and cell wall thickening.

KEGG pathway enrichment analysis revealed that the majority of metabolic pathways detected were the ones responsible for histidine, arginine biosynthesis, beta-alanine, cysteine, methionine, arginine, and proline biosynthesis dedicated to the metabolism of amino acids thereby revealing that the amino acid pathways were significantly enriched in wheat in response to *F. graminearum*. It was also found that FHB-resistant wheat activated shikimate-mediated secondary metabolism to inhibit *F. graminearum* growth and reduce mycotoxin production. The shikimate pathway is pivotal in the biosynthesis of various aromatic compounds, including phenolic acids and flavonoids, which have been shown to possess antifungal properties.

Enhanced production of these secondary metabolites in resistant wheat cultivars disrupts the pathogen's ability to colonize and produce harmful mycotoxins such as deoxynivalenol (DON). N-containing compounds are principally derived from aliphatic amino acids, polyamines, and purines as precursors. The biosynthetic pathways of N-containing compounds are remarkably diverse. The alkaloid class, for instance, comprises numerous families of compounds, such as tropane, pyrrolizidine, benzyloquinoline, and purine alkaloids. Phenolic acids, including p-coumaric acid, syringic acid, and ferulic acid, are considered to be significant phenolic acids found in our genotypes in response to the *Fusarium* pathogen.

A recent study examined the metabolic responses of wheat genotypes susceptible or resistant to FHB when exposed to *F. graminearum* mutants lacking trehalose synthesis (Liu et al., 2022). The metabolic analysis of wheat spikelets demonstrates that sugars play a crucial role in resistance to *F. graminearum* and DON accumulation. In our metabolome analysis trehalose was highly accumulated (10.52-fold) in *F. graminearum*-inoculated samples thereby inducing the FHB resistance. As a signaling molecule, sucrose plays an essential role in various plant metabolic processes and is vital for plant growth thereby activating the plant's immune response. Previous studies on the metabolomics of wheat leaf and stem tissues also showed that the levels of betaine, sucrose, glucose, glutamate, glutamine, alanine, trans-aconitic acid, and several aromatic compounds were positively correlated with resistance to FHB (Zhang et al., 2022). In conclusion, our observations indicated that the metabolites and their pathways detected in the study might be involved in the resistance mechanism against FHB disease in the resistant wheat genotype, Nobeoka compared to the susceptible wheat genotype, Sonalika. Further studies analyzing the roles of these altered metabolites may facilitate in devising of effective crop protection strategies against *F. graminearum* infection in wheat.

Chapter. 5

Summary and Conclusion

5.1 SUMMARY AND CONCLUSION

Wheat (*Triticum aestivum*, L) is an important cereal crop with high nutritional value and is consumed globally. Apart from the abiotic stresses, Fungal infestation, specifically by *F. graminearum*, causes a major cereal disease, Fusarium head blight, resulting in significant productivity loss worldwide (Goswami & Kristler, 2004). In addition to the mycotoxins, a no of candidate effector, non-effector, and other defence-related molecules are secreted by the pathogen to infect the host and to decide the type of molecular interaction. However, the virulence and pathogenicity mechanisms underlying these effectors released by the pathogens are still in an infancy stage. These effectors have diverse roles and are involved in suppressing host immune responses by manipulating host defense machinery (Gorash et al., 2021). However, the function of most of these putative effectors remains unknown. Moreover, these putative effectors expressed at different time points in different tissues in the hosts revealing their role in pathogenicity and virulence by orchestrating pathogenesis at different stages of infection in the hemibiotrophic lifestyle of the fungal pathogen, *F. graminearum*. It's opined that a conserved set of molecular players from both partners determines wheat's susceptibility to *F. graminearum*. Consequently, core effectors are a useful tool for wheat breeding and may help identify the essential host susceptibility variables that *F. graminearum* targets.

Therefore, it becomes important to understand the interactions between *F. graminearum* and wheat to combat the disease incidence on crop plants. Understanding the structural and functional annotation of *F. graminearum* effectors, along with gene expression studies during pathogenesis, has the potential to develop effective FHB control strategies. The current study is carried out to enable the identification of the candidate effectors and non-effector genes using a genome-wide search in the genome of the *F. graminearum* strain PH-1 involved in the compatible and incompatible interaction of wheat using integrated-omics approach. An *in-silico*-based genome-

wide search for putative effector genes revealed a total of 309 sequences, of which 243 were putative cytoplasmic effectors while 47 were apoplastic effectors. Further, the conserved protein domains/ motifs and 3D structures detected in their gene sequences through homology modeling confirmed them as putative effectors. A time course study to analyze the gene expression both at early and late stages of infection in two different host systems revealed that the majority of the effectors and non-effectors expressed in both the pathosystems. The bioassays were performed in order to test the virulence of these effectors in *Arabidopsis* and, in wheat, employing both susceptible and resistant wheat genotypes, Sonalika and Nobeoka, respectively, revealed that the majority of these putative effectors functioned as pathogenicity genes, while 11 of them might be involved in virulence.

Further, by selecting one of the important pathogenicity factors (FGSG_09908) from our study, we tried to assess the effect of siRNA-treated *CRP* gene on fungal morphology, sporulation, and total biomass in axenic cultures using RNAi approach. Also, to determine the effect of *CRP* siRNA-treated *F. graminearum* and *M. oryzae* on their respective host plants by SIGS. The study revealed a significant impact on silencing of the *CRP* gene as monitored through gene-expression studies, indicating the role of the *CRP* gene in reducing fungal infection and growth. Since *CRP* gene is crucial in cAMP-pathway regulation, which is supposed to be involved in fungal development and growth processes, *CRP* appears to be an appropriate gene to target the fungal pathogens to confer crop protection against fungal diseases. *In vitro* studies demonstrated that *CRP*-siRNAs treatment was effective at a 35 nM concentration. In planta treatment of *CRP*-siRNAs reduced the severity of *Fusarium* infection and blast symptoms in wheat and rice, respectively. Therefore, our findings showed that SIGS-mediated down-regulation of *FgCRP* gene expression resulted in inhibition of *F. graminearum* infection in wheat and *Arabidopsis* as well as *M. oryzae* infection in rice.

Our study revealed varied metabolomic responses between resistant (Nobeoka) and susceptible (Sonalika) wheat genotypes. The accumulation of both constitutive and induced metabolites was observed in wheat-*F. graminearum* interaction comprising flavonoids, amino acids and their derivatives, lipids, sugars, organic acids, alkaloids, terpenoids, and other classes of metabolites indicating that these metabolites and their pathways detected in the study might be involved in the resistance mechanism against FHB disease in the resistant wheat genotype, Nobeoka, compared to the susceptible wheat genotype, Sonalika. An unsupervised method utilizing principal component analysis (PCA) on key features successfully distinguished samples by the concentration of metabolites and identified metabolic markers associated with defense and disease resistance against *F. graminearum* TN01. The elevation of benzoxazinoids in our study indicates their role as defensive secondary metabolites in wheat genotypes following inoculation with *F. graminearum*. Further studies analyzing the roles of these altered metabolites may facilitate in devising of effective plant protection approaches against *F. graminearum* infection in wheat.

The salient points to conclude the research findings from the current study are as follows:

- Morphological, molecular, and phylogenetic analyses of the fungal strain isolated in this study confirmed TN01 as *F. graminearum* strain, which showed significant similarity with the reported *F. graminearum* strains. Detached leaf assay and histological studies in Arabidopsis and wheat genotypes revealed the virulent nature of *F. graminearum* TN01.
- A genome-wide search in *F. graminearum* strain PH-1 led to the detection of several putative effector and non-effector genes, of which 16 were selected for gene expression studies. The 3D structures of 6 effectors showed matches with some of the already identified effectors from other pathogens based on homology modeling using template-free modeling by TrRosetta.

- The findings also revealed a higher intensity of DAB staining, indicating elevated H₂O₂ accumulation in the leaf tissues of *F. graminearum*–inoculated Sonalika at 72 hpi compared with *F. graminearum*–inoculated Nobeoka. This could result from the necrotrophic phase of *F. graminearum* at a later time point of Infection.
- Gene expression studies in *F. graminearum*–inoculated Arabidopsis and wheat at early time points of inoculation showed up-regulation of putative effector genes in Sonalika, suggesting their possible role in pathogenicity. A few of the expressed candidate genes exhibited the potential to function as virulence genes (*FGSG_00060*, *FGSG_00061*, *FGSG_00062*, *TRI5*, and *TRI6*).
- The majority of these putative effectors functioned as pathogenicity genes, whereas a few of them might be involved in Virulence and were induced in wheat spikes in response to *F. graminearum*. The significant level and similar patterns of expression of the above genes at different time points indicate that these effectors exhibit both spatial and temporal expression in the host to facilitate pathogen infection and spread in the host plants.
- One of the genes (*FGSG_09908*) was induced in the FHB-inoculated Arabidopsis and wheat in response to the pathogen, suggesting it as a putative pathogenicity factor.
- The exogenous application of *CRP*-siRNA induced changes in *F. graminearum* and *M. oryzae* conidial morphology in comparison to the control. Deformed spores and mycelial growth were also observed in siRNA-treated *F. graminearum* and *M. oryzae* samples in comparison to the control and unrelated control (URC). siRNA resulted in the knockdown of the candidate effector gene *CRP* when supplied through liquid media, the same was confirmed by gene expression at the RT-PCR level.

- Significant reductions in the growth of aerial mycelia were observed at 35 nM and 100 nM siRNA concentrations. siRNA-treated *F. graminearum* and *M. oryzae* spores showed a significant reduction of infection on their host, wheat, Arabidopsis, and rice, respectively.
- RNA interference of candidate effector gene (*CRP*) by siRNA visibly inhibited the growth and infection of *F. graminearum* and *M. oryzae*, which can be targeted to prevent pathogen infection. Significant knockdown of *CPR* gene expression was observed at the mRNA level in both the hemibiotrophic pathogens against different hosts.
- A total of 809 metabolites were detected across all combinations. An increase in the level of amino acids, flavonoids, organic acids, inorganic compounds, terpenoids, fatty acid derivatives, and other related phytochemicals was observed in Nobeoka when compared to Sonalika.
- Amino acids, including proline and alanine, were shown to accumulate during FHB resistance.
- Principal component analysis (PCA) revealed an overview of the metabolic similarity or difference among the Resistant and susceptible sample groups & were distinguished into two groups by PCA plot analysis. The interpreted values of PC1 and PC2 were 60.6% and 18.5% in NT/ST samples, respectively. The results from PCA manifested that NT and ST samples have mutually distinct metabolic profiles.
- Phenylpropanoids, carbohydrates, terpenoids, amino acids, derivatives, and fatty acids in infected wheat heads have been implicated as potential contributors to FHB resistance. Our studies also correlated with previous findings suggesting their role in plant defense signaling, antimicrobial activity, and cell wall thickening.

- The metabolic analysis of wheat spikelets demonstrates that sugars play a crucial role in resistance to *F. graminearum* and DON accumulation. In our metabolome analysis, trehalose was highly accumulated (10.52-fold) in *F. graminearum*-inoculated samples.
- Antioxidants such as alpha-tocopherol, lipoic acid, and L-ascorbate were differentially accumulated in wheat in response to *F. graminearum*. Overall, metabolites of this category, such as flavonoids, indicated a higher antioxidant and ROS scavenging activity during *F. graminearum* infection.
- Pentacyclic oxylipins such as 16-oxo-palmitate, 3-oxo-palmitate, 18-oxo-oleate, and methyl jasmonate were abundantly observed in our study as well. The biosynthesis of methyl jasmonate (Me-JA) through the octadecanoid pathway initiates with linolenic acid, a metabolite that is enriched in *F. graminearum*-inoculated Nobeoka samples.
- The metabolic profiles revealed that Me-JA plays a vital role in plant defense against necrotrophic fungi at later stages of infection in wheat.
- The mRNA transcripts of *PR1* (*pathogenesis-related protein 1*) showed elevated levels in *F. graminearum* (Fg)-inoculated Nobeoka compared to Sonalika at all time points (hpi).
- *F. graminearum* TN01 infection resulted in elevated expression of the *PDF1.2* gene in Nobeoka as compared to sonalika.
- The Trichothecene genes *TRI5* and *TR6* showed significantly induced expression in both genotypes upon FgTN01 infection.

Future prospects of the research study

The study is elucidating the pathogenesis and virulence mechanism of the *F. graminearum* TN01 strain and the host defense mechanism underlying the interaction of wheat with the hemibiotrophic fungal pathogen by studying the role of different putative effectors through gene expression, RNAi interference, and metabolomic pathway. Further, the individual characterization of the effectors with host-resistant components may help in mitigating the FHB infection in plants.

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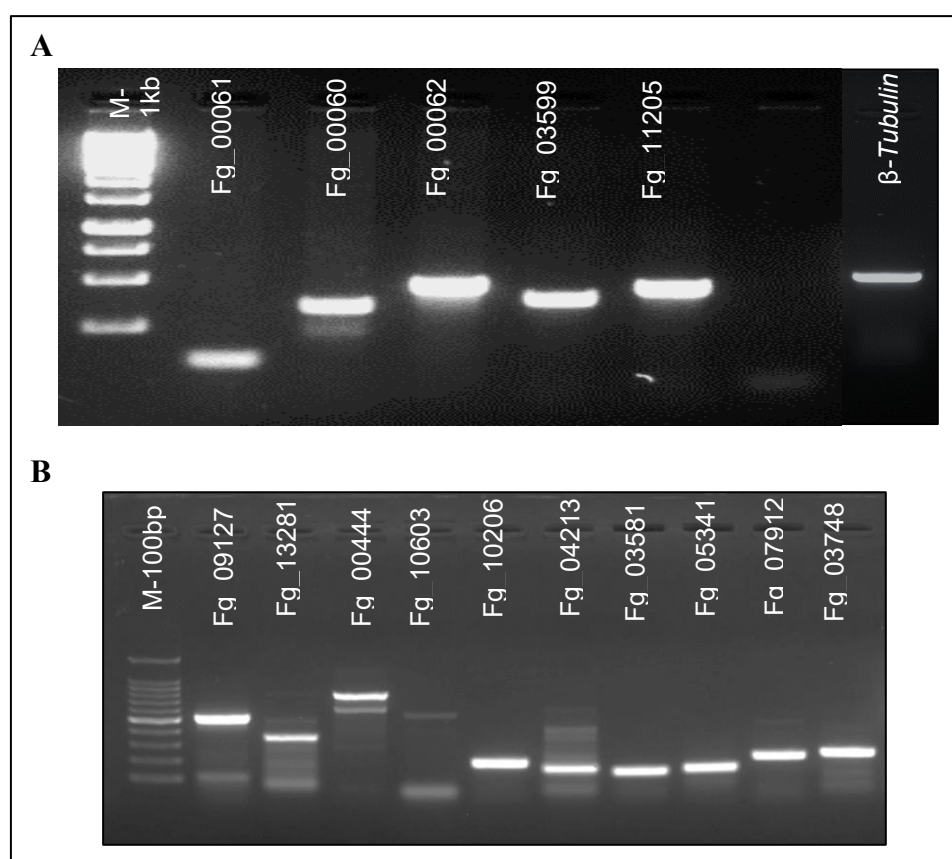
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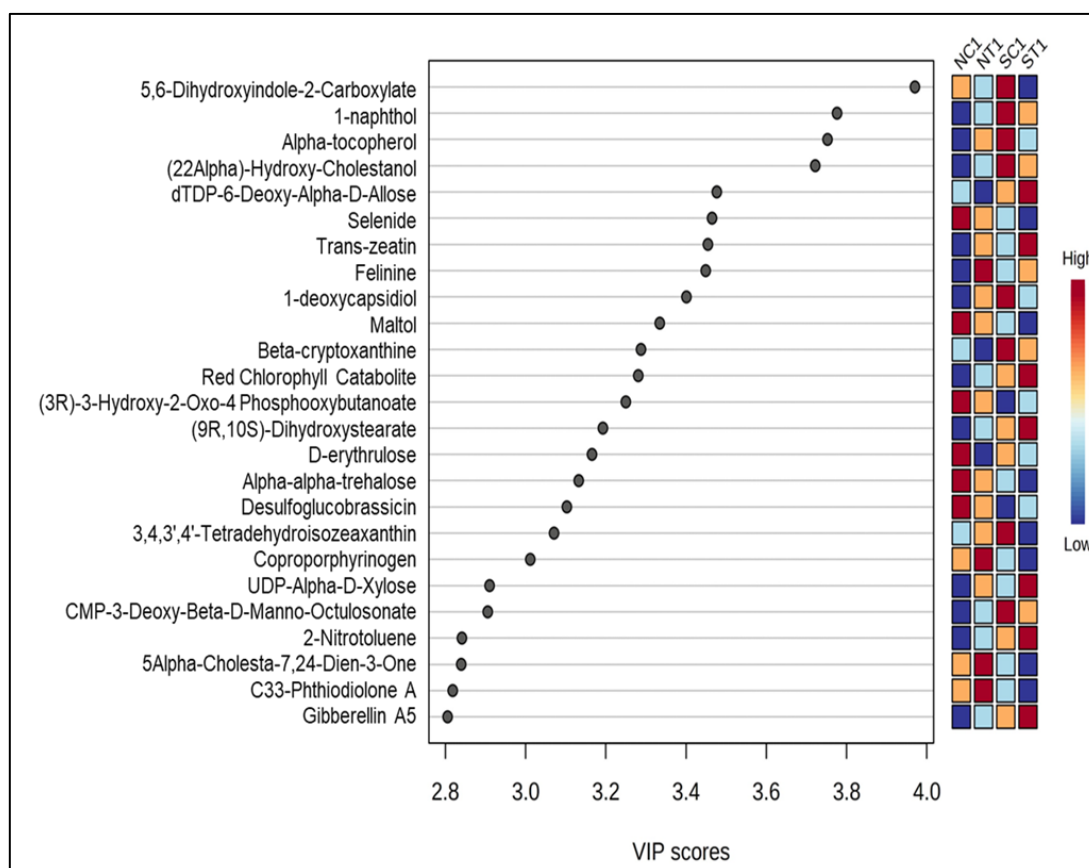
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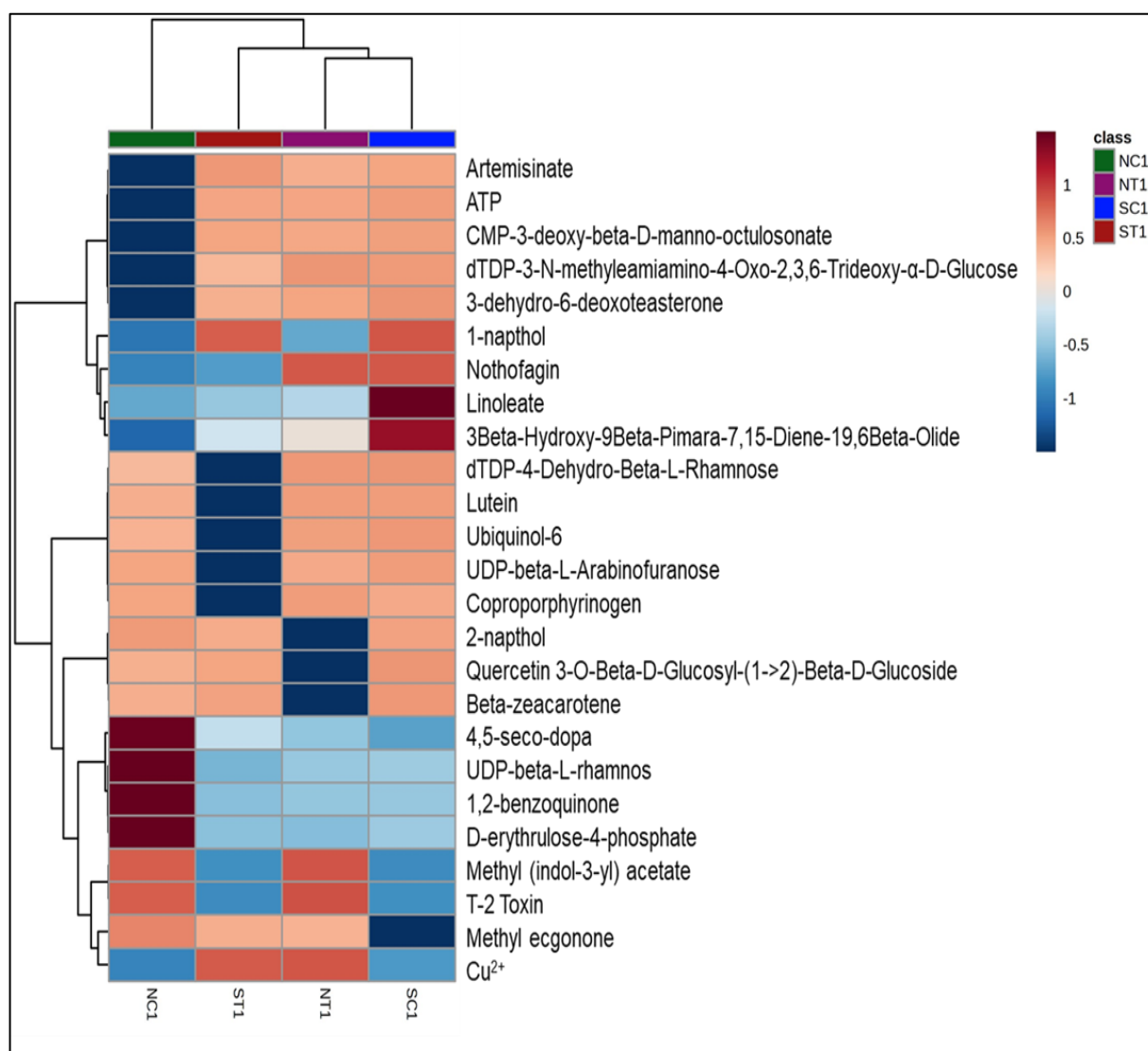
Supplementary Figures



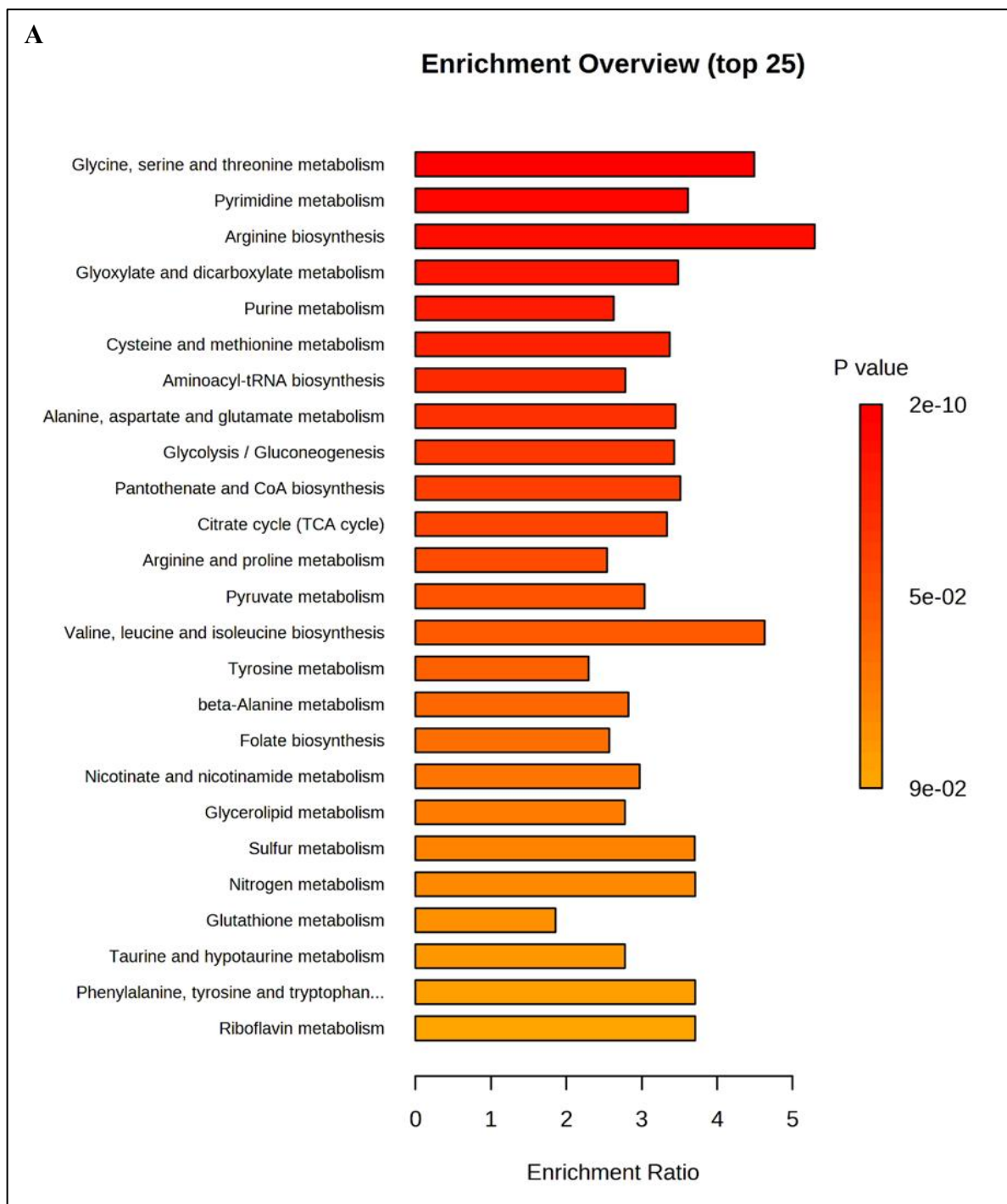
Supplementary Figure 1. Genomic PCR-based amplification of the putative effector gene sequences in *F. graminearum*. The genomic DNA of *F. graminearum* strain TN01 was used as template with gene-specific primers to amplify the corresponding DNA band size of each of the putative effector genes (A-B). DNA Markers: 1 kb and 100 bp.



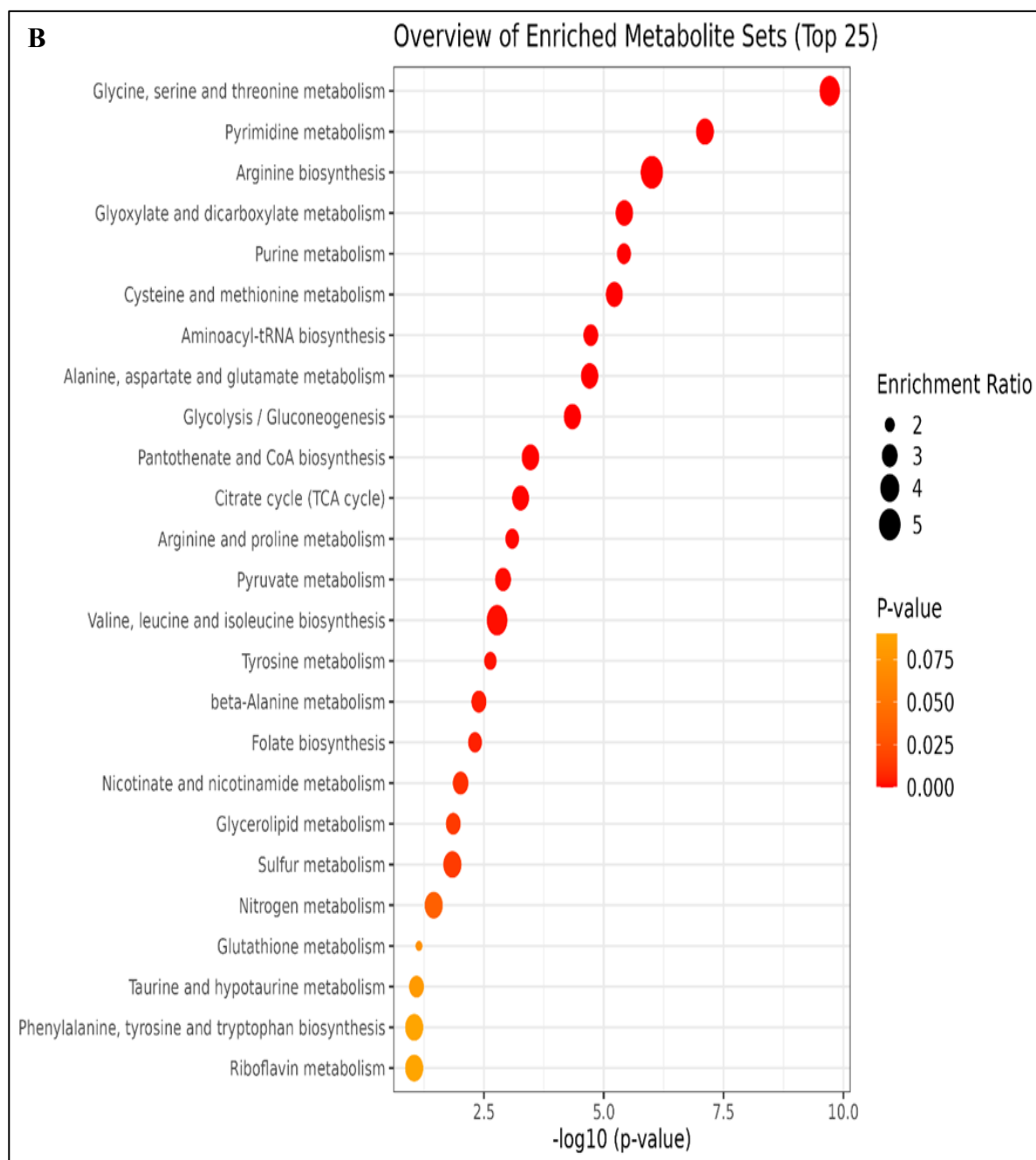
Supplementary Figure 2 Metabolite profiling based on VIP (Variable importance in the projection) scores using Partial Least Squares—Discriminant Analysis (PLS-DA). The abundance of metabolites in *F. graminearum*-challenged Nobeoka (resistant) and Sonalika (susceptible) wheat genotypes at 48 hpi based on Variable Importance in Projection (VIP). The differential visualization patterns among the four treatments (NC: Nobeoka-Control; NT: Nobeoka-Treated; ST: Sonalika-Treated and SC: Sonalika-Control) represent a distinct separation of metabolites between each pair of comparative samples (NC, NT, SC, ST). Metabolites with VIP scores ≥ 1 were deemed as affected by *F. graminearum* TN01. Colour bar indicates significant levels ranging from highly significant (red colour) to less significant (dark blue colour).



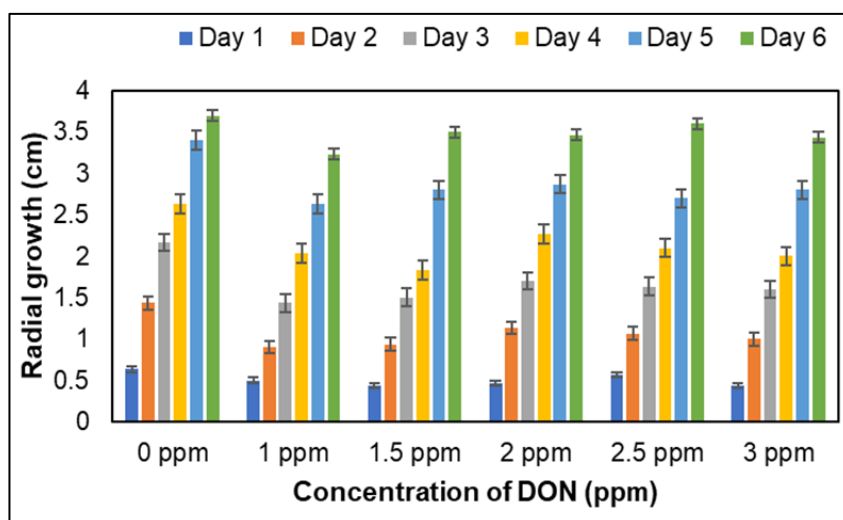
Supplementary Figure 3 Comparative metabolomics profiling and Hierarchical Clustering Analysis (HCA) of FHB-resistant (Nobeoka) and FHB-susceptible (Sonalika) wheat genotypes at 48 hpi. HCA was performed on the top 25 significant metabolites to illustrate variation in metabolic responses.



Supplementary Figure 4 (A) Pathway enrichment bar graph showing the top 25 abundant metabolites in FHB resistant-Nobeoka compared to FHB-susceptible-Sonalika challenged with *F. graminearum* TN01 at 48 hpi.



Supplementary Figure 4 (B) Pathway enrichment dot plot showing the top 25 abundant metabolites in FHB resistant-Nobeoka compared to FHB-susceptible-Sonalika challenged with *F. graminearum* TN01 at 48 hpi. The red-colored solid circles indicate higher significant levels compared to orange-colored solid circles.



Supplementary Figure 5 Radial growth inhibition of *F. graminearum* TN01 at different concentrations of DON treatment. Radial mycelial growth was monitored for 6 days and colony diameter was recorded for all the treatments against.

Supplementary Tables

Table S1. Details of primers used to amplify and sequencing of the internal transcribed spacer (ITS), D1-D2 region of the 28S region of nuc rDNA (D1-D2), and (*TEF1*) gene, along with annealing temperature, expected amplicon size, and accession number.

Primer Name	Sequence 5'-3'	Annealing T _m (°C)	Amplicon size (~bp)	Accession No.
ITS	F: TCCGTAGGTGAACCTTGCGG R: TCCTCCGCTTATTGATATGC	55	500-600	OR073525
D1-D2	F: GCATATCAATAAGCGGAGGAAAAG R: GGTCCGTGTTTCAAGACGG	55	500-600	MK814342
<i>TEF</i>	F: CAT CGA GAA GTT CGA GAAGG R: TACTTGAAGGAACCCTTACC	48	300	PP726712

Table S2. The nucleotide sequences of candidate effector genes of *F. graminearum* strain TN01.

S. No	Gene Name	Nucleotide Sequence details
1	FGSG_00060	>FGSG_00060, <i>Fusarium graminearum</i> Hypothetical protein (FGSG_00060), partial sequence ATGTATACTCTCCTCACTCTCACCACCCTTCTTTTCGCTGC CTCAGCACTCGGCATAAATTGCCGAGGATCAGGCTGGTG CAACATCAACAGCGCCAGCTTGGACAATGTTCTCACTAA AGCGAAACAACACTACAAGCCCGCGGACAAGGAGATCACC ACTGGGGAGAGGGCGTTCAAATTGCCTGCTCCGGCGGCC AATGGGGATCTATCTGCGCCTTCTTCCAAAGCGGCGCTA GCGGCAACACGGATCGGGCCGTCGAATTGATTCAGGGCA TTCTCGACCACGGTTGCACTCAGTGTGGTAGTATTCCCAC TGAAGAAGGCAATAACGTTGATAACGGACAGCTCACTGT CAACTATGTTGAGAACCCTTGCTGCGATGGCGAATGTTA CTGCCCTATCTAA
2	FGSG_00061	>FGSG_00061 <i>Fusarium graminearum</i> Hypothetical protein (FGSG_00061), partial sequence ATGCATTACTCTACTCTTACTACCCTTTCCGCTCTTGTCAC CATGACATCCGCTCTCGGAATCAACTGTCGCGGCTCAGG ACTCTGCTCTGGTGGCGCCGGCAACCTCATCAATCTGAA AGCAATTGTCGACAACATTCAACCACGAGACCGCCACTA TGAGACAGGCCAGCAAGTTGCTTGTACTGGTGACACTTG CGCTTTCTTTCAGAGTAGCGCAACCGGCACTGCTGATGAT GTGTCCTGGGCCCTACAGGCCCTTCTTGACCACGGCTGTA AGAAGTGTGGGTCTGTCCCCATGCAGGATGGCAACAACG TCGATGATGGGCAGTTGACAGTCAACTATGTTCAGCGACC CTAGTTGCCAGGGAGCTTGTTAA
3	FGSG_00062	>FGSG_00062 <i>Fusarium graminearum</i> Hypothetical protein (FGSG_00062), partial sequence ATGCACTTCTCAACATCAATTCTTATCTTCACCTCCACCG TCGCAGGTTTAGGCATTAAGTGTCTGTCGTCAGCGGCTTCTG TGACCTCACCTTCGGCTCTAGCATTGGAGATATCCAGGA CCAGATCGGCATCCTCGTTGCCGATGGTCAAGGCGACAG ACACTTTGACACCGGAGCTCAAATTGCTTGTACCCAAGG CAACCAGGGCAGTATCTGTGCTTTCTACCAGAGTGGTGC ATCTGGGTCAGCGAGAGACGCATTTGGTCACATCCAGCA GATCATCGACCACGGGTGTTCACTTTGCGGCAGTGTTCCG ACACAGGAAGGAAACAATGTGGACGATGGGCAGCTGAC AGTCAACTATGTTAGCGACCCATGCTGTGAGGGGAATTG TCATTGTTAGATCTTTTCATGTATTTAGGGAACCAAATGT ATCTCTTGAGTAT
4	FGSG_03599	>FGSG_03599 <i>Fusarium graminearum</i> Hypothetical protein (FGSG_03599), partial mRNA GTCATCAATCCAACACACAAACATCCATATCATTCCATC CCATCAGTAAACATGCAGTTCACCACCTCCTTCATCGTCG CCGCTTTCCTCGGCACTGCCTTTGCCATGCCACAGGCCGC ACCAAGTTGACTGCCCCGAGACGTCAGCCATTCCCACCTG CGGTGCCCTTGATACCTCTGCAGCTGCAGCTGCTGGA

		TGCACCGACATTGCTTGCCAGTGTGCTAGCTCTGAAGTG ATTCAGGCTTCAGCTCTTGGCTGTGTTGTCGGTAACTGTG GAATTCCCGTGGCTCTATCAGTGCAAGCTGCCGCCGCTG CCGTCTGCACTGCCTGTGCTTAGATGTGGTATATTTATTG GGACATATAAGTGACATTATATACCTGAATACAAGCTAT ATTAGTTGCGG
5	FGSG_11205	>FGSG_11205 <i>Fusarium graminearum</i> Hypothetical protein (FGSG_11205), partial mRNA ATGCAGCTGACCAACCTCTTCTGTCTCGCCAGTGTCTGA CCTCCGTGTCAGCAATCACCGTATCCTACGACCCAGGCT ATGGCGAAGCTGGCCGCGCCATGACAGCCGTCTCCTGCT CCGATGGCACAACCGGCTCATCACGCGCTACGGCTGGA AGACTCAAGGCCAGATCCCCAAGTTTCCCTACATTGGCG GCGCGCAGGCCATCGCTGGCTGGAAGTCCCCGAGCTGCG GCACGTGCTGGAAGCTTACCTACAAGGGCAAGAGCATCA ACGTCTTGGCCATCGACCACACTGCTGCGGGCTTCAACA TTTCTCCCGCGGCGATGAACGCTCTTACCAACAACCAGG CTGTTCAAGCTTGGTCGGGTTGATGCGACTGCTACTCAGGT CGCTGTTAGCAACTGTGGATTGAAGAAATAG
6	FGSG_13281	>FGSG_13281 <i>Fusarium graminearum</i> Hypothetical protein (13281), partial mRNA ATGGATGACGCAGAGCTTGATCAGATCCGAAAGGCTCGT CTAGAGCAGCTCAAGGCCCAAGGTGGCGGTAGTAGTGGA GGTGGTGGCTCCGGGCAAGAGCAGCAACAGCGCCAACA ACAGCAGCAAGATGATGCTCGTCAACACGTCTCTCAACCA GATCCTTCACCCCGAAGCCGCGGACCGTCTGGGCCGCAT CCGTCTCGTCAAGGAATCGCGCGCCGCGACATCGAGAA CCGACTCATCACACTTGCCCAGACGGGCCAGCTGCGACA AAAGGTCACCGAGGCGCAGCTCAAGGAACTTCTCAACGC CATGTCTGAGAGCAAGGAAGAGGAGAAGATTGTCGTGA GCAGGCGCAAGGCTTGGGATGACGATGATGATTGGACT TGTA
7	FGSG_10603	>FGSG_10603 <i>Fusarium graminearum</i> Hypothetical protein (10603), partial mRNA ATGCCTTCTCCCAAGAATACCTGCTTTATTCTTCTATCTTT CCTCGCTCATAGCGCAGTAGCCACTCCTTCTGGCCGAAGT CCAGAGAGATCTCTCCAAGATATTGACACCGTTGTCTAC ATTTCGAGTGATGATTCTGGTTTCTACAAATCTCAGGACA TGACCCAAGCTATCAAGGTCAAACAAGCAGCCCTTCCAC TACACAAGCGGTCAAGTGCCAAGGTTGAGGCCTGTTTCT CGCCCCAGTGACAACTGCAGAGTTATCTACGACGGTA GCCTCAGCAGCAATTCGCCGTGCGTTCAAGCCGTCAACA CGGCCTGTCTTATTGTATCAAACCTCAACGACGCAAAGA TCCAGGCCTGGAACAAAGCGCAATGCAACGGAAACAAG TCCACCTTCCGTGGCTGTGGTGCGCGCACCAACTTTGCTG CTCCTGGTACCAATTCCATTGGTGTTACATAGGCTGCAG CTGA
8	FGSG_07921	>FGSG_07921 <i>Fusarium graminearum</i> Hypothetical protein (07921), partial mRNA

		ATGCACTGCCTTCTTCTCCTGACCGCTGCGTCTCTCGCTG CGGCTCACGTTCTTTCAGTCAGCGTGCCCGCATGTCCCAA ATACGGCACCATCAAGTTTTCCAAATCTGTTCCCGACGGT GATCCTTTCCCGCGTACCGAAGTCGACCTCTGCTACACAG ACACCGCTTTGTCCCTACAATTACGGCCTTTGACGAGAA ATACTTCTACTTTAACGAATCCCAAGGAACCAACGACGA TATCTGGGCTTACGAAGTCGTTGAGGCGTTCATCTACAA AGGAACCGACGATCCGCAGACCTATCTCGAGTACGAAAT CAACCCCAACAACGTAACCTACCAAGCCTTTGTCTACAA TCCCTCCAAGGTTTCGAGCCGAGGGCGCTCCGTTTCGACCA TTTCTTCATCTCAGACCCTGAAGGCGATGGATTCACGGCA GAGACACAATAAACAAGCGGGCGAAAAAGTGGGTGAG CAAGGCACAGATCCCGCTGGGTCTGTTCAACGTCGATCC GGGATGTGCATGGGGTACAAAGTGGAGGATGAATTTCTT TCGCACGGTTACGAGCCCCAAGATGTTTCCTGACCAAGA CCTTGGTGGCTGGAGCTCACCAGATAAAGCCAGCTTCCA CATTACGAAGTTCTTTGGTCATGTCAACTTTGTGTAG
9	FGSG_10206	>FGSG_10206 <i>Fusarium graminearum</i> Hypothetical protein (10206), partial mRNA ATGAAGTCCTTCTCCCTGATCACTCTCTTTTGCACCCTGG TCACTGCTGCAGTCATCCCTCGAGATACTGTCTTTGACGG CCACTGCTGCTTCACTCTACATGACGTAGCCAACGATGC AGCAGTGCAAGAAAACAGCAATGGAACTTGCTCCTCAA TGCTGGCAGCACCAAGTGGCTTCTTCTGCATTGACCTGTCC AACTCGCAAAACATTCTGCGAGACCGCTCTTTCAATGCCT GCTTCCTGAACCCTAGCGGCGAGGTTAAATGCGTCGACC CAACACCCGGCTTTGAGTCTTGGACCTTGAAGAAGAGCG GCAGCAACACTCTTCTCCACCGCGACGGTGCAACCGCTT TCAACTCATGCTCCAAGACTTCAACAAGTTCAAAGGGAA CGGTGCTATTTGGCGACAAGCACACTGGTGCTTCGACGT GCAAGAAGACTACACTCAAGGTGAAGAATTTGAAGGGG ACATGCAAGAAGTTTGA
10	FGSG_03748	>FGSG_03748 <i>Fusarium graminearum</i> Hypothetical protein (03748), partial mRNA TCTGGTGACAATGTGCGATACAACCTCGACACTTGCAAAG GACTACATCTGCGGAGACAAGCGTCTTGGCCCTAGTCGT CTCCCAACCAAGCTTCCCCTTGGCACCTTCGTCACTGGGT ACGACCGCTTTGGAGGACTGTCTCCAAACTCCTTCCTCGG GAAGTGGTACAACCTCCACCGAGGGGCTGATGGCCGCGAA GCAGGATGGATCTACCCCGAAAAGTACGGCTTCGAGCTT GAATGTTGTTCAAGTCCATCTTCATCTCGTCCCTCGCGAC TAGTGCTCTGGCCATTTACCTACCCGAAAACCATTAACCTG GTTAGAGACAACGCGGCTGCGCCAGGAGTAAAATGCGA CTGCTCTGGTGACAATGTGCGATACAACCTCGACACTTGC AAAGGACTACATCTGCGGAGACAAGCGTCTTGGCCCTAG TCGTCTCCCAACCAAGCTTCCCCTTGGCACCTTCGTCACT GGGTACGACCGCTTTGGAGGACTGTCTCCAAACTCCTTCC TCGGGAAGTGGTACAACCTCCACCGAGGGGCTGATGGCC GCGAAGCAGGATGGATCTACCCCGAAAAGTACGGCTTCG AGCTTGACGAAGAGAAGCTCCCCATCAAGGCCAACGTGG

		ATCTGATGCCCCGGTACACTTGTTGACCGTTTCGGCTATAA CACTGGTCGTTACATCTCCCCTGCCACCGCCCCCTTCTCC CAGAGAGCTCTCCACCCCCAGAACCTCGACAACGATGTG AACAAAGCAGTTCCCAAACAACCTACCACGTATACAACGTG AGCAGGATGTTCACTGTCCAGGCTGGACCCATCAGGCCT TGGTTCGGCCAGCCAGGCTTTGGAGTTCAATTCTTCCTCG GCAACGGCATCAGCGTGAAGGATTACCTCGACAACGGAC ACTTGGTTGAGCTGAAGCCATCTGATCTGCTCAAGGACG GAACTGGCTGTGGCTTTGCGCGTGAGGGTGATGATTCGG TCTCAGACGAGCTGTAG
11	FGSG_05341	>FGSG_05341 <i>Fusarium graminearum</i> Hypothetical protein (05341), partial mRNA ATGCGTTTCGTCAACCTCATCACCGTCTTCGTCTGGTCTCG CTGCCGCTGCGCCTGCGCCTGCTGTTGCACCTGTTGCTAC GCCCATAAAGATGATATGATCGCCGTCACGACTGTAGC TCCTACCGCCTCTGGTGATCCCGTCACCTACTGGTCCGCC AGCCCTGAATCGGTCAAGGTCTGCGATGAGGACACCTAC GTCGGTCGCGAGGGGCCCAAGGAGATGAAGCGTGCCAA CTACCGCGACTGTGCTGCGCTGCTCTCCAGCTTTGGATCC CGCAACGGTACTTTCTTCGTCCCCCGCGCTTGTGAAGACG ACAGCGAGTATGGTCTCAAAGACGGCCACGTCGATATCG TCAAGTCTGGTTCCTGCGCGTTTTCCGTCCGCGCCGATGC GGGATTGATGGTTGGCGATGAGGACATTGTCTGGATCAT GCGAAAGGCGGTTCTCAACAACCTCTGATGGGGAGATCCA CGGGATTGAGTCAACTGGCTATTAA
12	FGSG_04213	>FGSG_04213 <i>Fusarium graminearum</i> Hypothetical protein (04213), partial mRNA ATGCAATTCTCAGCCTCGTCCTTATGGCTACCTCAACCC TGGCCATCCCCGCCAAGCTACCTCCAAGGCCCCAGCAC CAACATGTATGGAAAAGGGCACAAAGGTTTCTGAATGGA AACTCACAGACTTCGTCTACAACGTCCAGAAGAGCACAA ACACCGCCACAGTCGAGTTTGACCTCCACAACGCCGCTC TCGACTACACGGCCAACTGCTACGGCAAGTCAGTCTCCA AGAAGGCCCTCTTTGACGGCAAGACCGACTACACCTGCA AGGTCCCCAACGGAGCCGACTCTGCCACCTTCAAGTACA ACCACAAGAAGGGCGTCATCTCCATCTTCCAGCACTGGG CCTGCGTCCAGGAGGGTGGTTGGTTCGAGGCCATTGGCA ACAGGACTTCTACCGAGACCAAGTCTAACTGCCACTCTA CGACCAAGTCCGAGACTTGCACAAAGGCCTCCTTCACGG TCCCCGTCTCTCGAGATGCGCGCAGTCTTGTA
13	FGSG_09127	>FGSG_09127 <i>Fusarium graminearum</i> Hypothetical protein (09127), partial mRNA ATGCAGATCTTCACTCTCGCCACCCTCCTTGCCCTCATGG AGATTGCCTCGGCCAAGCTTCACAACCAGGCCGTTTGCG TCACCAACCGCAAGTACGCTTCCAACGGAGGAACTCCCT TCAGCGTCAGCTACAACCTGGCGTGAGAACTACGAGATTC TTCCCGACGCCACCAGATGCGCTTGCGACTACTACAGGA ACCGCAACACTGGTAACAACCAGTGGGATAAGTGCCCTG ACTGCAACTTTGATGGTCTTGTTTGCGGGTCTCGGGACTG

		GCACTTGGCGGTGATGAGTTCACCTACTACTGCGAGAAG AAGTGTGGTGCCAGGGAGCTGAGGCTAACTAG
14	FGSG_03581	>FGSG_03581 <i>Fusarium graminearum</i> Hypothetical protein (03581), partial mRNA ATGCCTTCCCTCATCAACACTTTTGCTGCTCTGGCTTCCCT CTTGGCCATCTCCAGCGCTGCGCCCACTATTGTACCCCGC GCCACAGAGTGCCCTCCACTGGAAAAGCTCGCCTCCAA CCCTCGGCTCTCTACAACATCTTCCCCAGCGCTCCCAACG TCGCGAAGAAGACGTCTGGCTTCCACGTCGAGACCTACA ACAACGCCTCCCAAGTCGAGCAGCTTCTCGTTTTCAAGG ATCTCCCTGCAGACGCCAAGGACTGCAGCATCGGCTGGG CCCAGGGCGAGCGACCGGAGCGCACCTTTATCGTCAAGG GCGGCGATGCTCTGACCGAGATCAAGCAGCTATCTGGCT TCCACGATACCAAGTCAATCACGTACGAGACAGCCAAGA AGTTTGAGACGGCCGACAAGACCGCTGGTGCTGCCGACT TTACCAACTGGGACGATCTCCCTGCCCAAGGCCACATCA TCGGCGCGATCGACTGCAAGAGCTCCATCTACCTCAAGG CAGCTCTTCGAAACCCCGACGGCAACACAAAGGTCTACC TTGAGCAGAACAGCAAGAACGGACTGTACATTGAGTACA GCTGCTAG
15	FGSG_00444	>FGSG_00444 <i>Fusarium graminearum</i> Hypothetical protein (00444), partial mRNA GCTATTACTACTACTTCTTCTTCAGCCGTGCACCAACTCG ACTTTACCTCAAAACCTCATCCATCCCCAGCTCTACCACT CGAGTCTTTCTCATAGAAACCATCGCAATCCTAAACACTC ACATACATCATGGCTTATAACCGTCCCTACGACGAGGAT GCTCTCCCCAGATTGCGCGAGCCTGAGCAGAAGCCAGGA CAGTCCAGGACTCCAGCCCCGCAGCAACATCACCTCCT CCTCAGCAGCAGCAGTACCAGCATCACGCCCCCTCCTCCT CAGCAGTATCACCAGCAGCAGTCTCGATACGATAAGCCT CTACCAACCCAAAGAGATGCGCGATCTCATTCACTAGGT CAGGGCCCAGCTTCTCATGGTTACATGTCCCCGCCTCCAA AACTGGCGGCGCGAGACCCAGGCTCACAACCGTCCCG CCCCAACTCACGACCTCCTCCCTCGCCTGCTCTCGATGG CAATGGCTCCGATCCCACGCTCCTACCTCTATTCCGTGCT GTAGACAAAGACGGAAGTGGCCATCTTTCTGAGAGAGAA CTATCCGCGCGCTTGTCAATGGTGACTGGACCGCCTTTG ATCCTCACACTGTCCGCATGATGATCCGAATGTTTGATTCT TGATCGAAGTGGCACGATCGGTTTCGAGGAGTTCTGTGG TCTGTGGTCCTTTTTGGCTTCGTGGAGAACTCTCTTCGAC CGATTCGACGCCGACCGTAGCGGTAACATCTCACTCTCC GAATTCAACAACGCTCTCGTCGCTTTCGATATCGACTAA GCCCCAGTTTGTGCAACTGATTTTCAACACCTACGACAA GCGAAATGAAGGCGTCATGAGCTTCGACCTGTTCTGTTCA GAGCTGCATCTCTCTCAAGCGCATGACCGATGTCTTCAA GAAGTACGATGACGACCGCGACGGCTACATCACCTTGAG TTTCGAGGACTTTTTGACAGAAATTCTCAAGCAGCTCAA GTAAATAACGGAAGGCGTCAGGACTGGACAGTTTAAA GATGGGAAGAGAGTACTCGGTGCCGGAGCAAGCACTGG TGCTTCGAAACGTCATATGGATTATTTTTTGCATCCTGA

		GGAAAAGGACATGCATGTAAGGCGGATTTTACGACAGAT ATAAACCCAAGCCGGATGCTTCTGAGTTGATGATATGGG ATAGCATGTACCAAAGATACCCTAGGTCAAGTCTCCGGA TCCTGGCTTAGAGACCGAACTTACCTTGACATCAGACA TGAATTATGTTACATAAA
16	FGSG_09908	>FGSG_09908 <i>Fusarium graminearum</i> Hypothetical protein (09908), partial mRNA ATGTCCGGCCCTTTCTCCAGTCCCTTTGGGGAGAATAGCA ACCCCTTTGGAACAGACAGGGGTAAGAGGACAAACAAT GCAATCCATCGTGTTGTTGAGGAAGAGGAAGAAAACGAC ACCATCACATCGCCTACGAACGCTCGTTTCGGAGCATCA CCAAACTCTGCCTCCATGTTTTCGGGTCCGTTTGGCGGGG GCTTTGGCGGTGACTCTTCTGCTGAAGCACCTTCTGCGTC GCGAAACGTCCCAAACCCTGATAGTTACCCGGCCCCAGTA CAACTTCGGTCGCCGAAGTTCAGTATCTGCGGAATCTTTG AAACCCAGTGCCGATTCCTATGATAATTGGTCACCCCCTT TTCATGAGAAGACCCCGGACCAGATCGAACGTCTCAAGC ATGCCATCGAAGGCAATTTCTCTTCAGCCATCTGGAGG ATGAACAGAGCGCTCAGATTTTGGGTGCCCTTGTGCGAAA AGCCGATACCAGCCAAGGGCATTAAAGGTAATCAGCCAGG GAGATGCTGGCGACTACTTCTATGTCGTCGAGAAGGGAT CTTTTGACGTATATGTCAATCCCAGCGGCTCGCTGCAGCC CGGTCCCGATGGTATGGGTAACCAGGTTGGTAACATCCA AGCTGGTGGCTCATTCGGTGAATTAGCGTTGATGTACAA TGCCCCCTCGAGCTGCGACTGTAATTTCTGCCGAGCCTGGC TGCACTGTGTGGGCACTCGATCGTGTTACTTTCCGTCGCA TCTTGATGGAGTCGACCTTTGCTCGGAGACGCATGTATG AGAGCTTCCTCGAAGAAGTGCCTCTCCTTTTCGACCCTAAA CCCATACGAGCGATCCAAGATTGCGGATGCGTTGGAAAC ACAGAAATTTGCTCCAGGAGAGATCATCATTAAACGAGGG AGACCCTGGCCATGCGTTTTATCTGCTGGAAAGTGGCGA AGCGGATGCATACATTGGCCAGCCTGACAATAAAGTCCG TACTACAAGAAGGGTGACTACTTTGGTGAGCTTGCCTT GCTCAACGACGCCCCGCGAGCAGCTAGTATCGTTGCTGC GTCACCAGTCAAGGTTGGAAGCTTGGGAAAGAATGCCTT CCAGCGACTGCTGGGTCCTGTGCAAGGTATTCTACGAAG GACCAAGTATCAAGGTGTTGCAACTGGTGTTGAAGAGAT GGATCCACTTCACACCGGCTAG

Table S3 The consensus motif sequences detected in the effector genes of *F. graminearum* characteristic of their gene structure.

Gene Id	Consensus Motif Sequence
FGSG_00060	[IL]Q[AGQ][IL][LI]DHGC[KST][KLQ]CGS[VI]P[TM][QE][ED]GNNVD[D
FGSG_00061	N]GQLTVNYV[SE][DN]P[CS]C[DEQ]G[ADN]C
FGSG_00062	LGINCRGSG[FLW]C
	QGD[HR]H[FW][DG][ET]G[AV]QIAC[ST][GQ]G[NQ][QW]GSICAF[FY]Q
	S
FGSG_00444	Q[HE][HQ][APQ][PQ][PR][QP]QQ[QY][HQY]
	[MVW][RT][DMT][LMV][FI][DKR][KMR][FY]D[ADS]DR[SD]G[NTY]I[G
	ST][LF][SE][EF][FE]
FGSG_13281	Q[HE][HQ][APQ][PQ][PR][QP]QQ[QY][HQY]
	E[DK]IV[VW][IS][MR]RK
FGSG_03748	DGR[EV][AM]G[GW][IL]Y[PW]E[IK][HY]G[FI]E
FGSG_05341	DGR[EV][AM]G[GW][IL]Y[PW]E[IK][HY]G[FI]E
	E[DK]IV[VW][IS][MR]RK
FGSG_04213	[FH][VK]YN[VHP][SKQ][KR]
FGSG_07921	EKY[FL]Y[FI]
	[FH][VK]YN[VHP][SKQ][KR]
FGSG_11205	[TC][CV][WK]K[TL]T[LTY]K
FGSG_10206	[TC][CV][WK]K[TL]T[LTY]K

Table S4 Metabolites detected in wheat samples challenged with *F. graminearum* TN01 based on KEGG classification.

Pathway involved	Compound
Flavonoid biosynthesis	Quercetin, Apigenin, Vitexin, Leucocyanidin, Isoliquiritigenin, Myricetin, 2',4,4',6'-Tetrahydroxychalcone-4'-O-glucoside, Quercetin-3-O-beta-D-glucosyl-(1->2)-beta-D-glucoside, Kaempferol-3-O-beta-D-glucosyl-(1->2)-beta-D-glucoside, Chrysoeriol, Quercetin 3,3',7-trissulfate, Isovitexin, Apigenin, Quercetin 3,3'-bissulfate
Phenylpropanoid biosynthesis pathway	L-Phenylalanine, L-Tyrosine, trans-Cinnamate, Sinapate, Coniferyl alcohol, Coniferin, Spermidine, 4-Coumarate, Sinapine, 1-O-Sinapoyl-beta-D-glucose, Sinapyl alcohol, Sinapoyl malate, Sinapoyl aldehyde, Eugenol, N1, N5, N10-Tricoumaroyl spermidine, N1, N5, N10-Tricaffeoyl spermidine, Coniferyl acetate
Alkaloid biosynthesis	L-Tyrosine, 3,4-Dihydroxy-L-phenylalanine, Tyramine, 4-Coumarate, Morphine, Secologanin, (S)-Reticuline, Dopamine, (S)-N-Methylcoclaurine, Protopine, 6-Hydroxyprotopine, Dihydrosanguinarine, Salutaridinol, (S)-Norcoclaurine, (S)-Coclaurine, Codeinone, N-Demethylnarwedine, Glyoxylate, Xanthine, Caffeine, 7-Methylxanthine, (6S)-Hydroxyhyoscyamine, 6-Amino-2-oxohexanoate, Pseudotropine, Nicotine, Tropine, (S)-2,3,4,5-Tetrahydropyridine-2-carboxylate, Nicotinate, Putrescine, L-Phenylalanine, L-Lysine, L-Isoleucine,
Terpenoids	Geranylgeranyl diphosphate, Taxa-4(20),11(12)-dien-5alpha-yl acetate, Taxa-4(20),11(12)-dien-5alpha-acetoxy-10beta-ol, Baccatin III, Momilactone A, Gibberellin A3, Gibberellin A12 aldehyde, Geranylgeraniol, Gibberellin A20, Gibberellin A12, Gibberellin A24, Gibberellin A9, Gibberellin A4, Pyruvate, D-Glyceraldehyde 3-phosphate, 2E,6E)-Farnesol, Geranylgeranyl diphosphate, R)-Mevalonate, 2-Phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol, 4-Coumarate, trans-Cinnamate, Chorismate, Phylloquinol, 2-Phytyl-1,4-naphthoquinone, beta-Tocopherol, gamma-Tocopherol, (S)-Limonene, (-)-trans-Isopiperitenol, Loganin, Secologanin, (-)-Isopiperitenone, Deoxyloganin, 7-Deoxyloganeate, S)-2,3-Epoxysqualene, (2E,6E)-Farnesol, Nivalenol, Capsidiol
Amino acid metabolism	L-Glutamate, Glycine, L-Alanine, L-Lysine, L-Aspartate, L-Serine, L-Methionine, L-Ornithine, L-Phenylalanine, L-Histidine, L-Proline, L-Threonine, D-Lysine, D-Proline, D-Cysteine, N-Acetyl-L-glutamate, 1-Pyrroline-4-hydroxy-2-carboxylate, LL-2,6-Diaminoheptanedioate, S-Adenosyl-L-homocysteine, L-Aspartate, L-Ornithine, L-Tryptophan, L-Leucine, L-Histidine, L-Isoleucine, Homoisocitrate,
Plant hormone signal transduction	Salicylate, Indole-3-acetate, Dihydrozeatin, Abscisate, (-)-Jasmonic acid, Gibberellin A4, (-)-Jasmonoyl-L-isoleucine,
Phenylalanine, tyrosine, and tryptophan biosynthesis	Phosphoenolpyruvate, L-Tryptophan, L-Phenylalanine, L-Tyrosine, Anthranilate, 5-Phospho-alpha-D-ribose 1-diphosphate, Chorismate, Prephenate, D-Erythrose 4-phosphate, Quinate, L-Aspartate 4-semialdehyde, Indole, Shikimate, L-Arogenate, 5-O-(1-Carboxyvinyl)-

	3-phosphoshikimate, 1-(2-Carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate, Shikimate 3-phosphate, 2-Amino-3,7-dideoxy-D-threo-hept-6-ulosonic acid
Benzoxazinoid biosynthesis	HBOA, DIBOA, DIBOA-glucoside

Identification of molecular determinants involved in compatible and incompatible interaction of wheat- *Fusarium graminearum* using integrated-omics approach

by MASSARAT FATIMA

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Professor, Department of Plant Sciences,
School of Life Sciences,
RAGIBA MAKANDAR Ph.D
Professor

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School of Life Sciences
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