T.R.
EGE UNIVERSITY
Graduate School of Applied and Natural Science

A PRELIMINARY STUDY ON SPRAY INDUCED GENE SILENCING TECHNOLOGY FOR PROTECTION AGAINST GREY MOLD AND FUSARIUM HEAD BLIGHT DISEASE

MSc THESIS

Reemana FATEMA

Department of Seed Science and Technology

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Reemana FATEMA

Supervisor: Assoc. Prof. Dr. Evren KOBAN BAŞTANLAR
Co-supervisor: Assoc. Prof. Dr. Ramesh Raju VETUKURI

Department of Seed Science and Technology
Erasmus Mundus Master Program in Plant Breeding Second Cycle Programme

İzmir
2021

Here we certify that this thesis entitled “A PRELIMINARY STUDY ON SPRAY INDUCED GENE SILENCING TECHNOLOGY FOR PROTECTION AGAINST GREY MOLD AND FUSARIUM HEAD BLIGHT DISEASE” conducted, prepared and submitted for the degree of Master of Science by Reemana FATEMA has been recommended for acceptance and approval for oral defense as it complies both with the “Ege University Graduate School policies” and “Ege University Graduate School of Natural and Applied Sciences' rules and regulations”. This study is fully adequate, in scope and quality, as a thesis for the degree of Master of Science and the Candidate was found successful and the thesis was approved by the thesis committee unanimity/majority voting on 21 / 09 / 2021.

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I hereby declare that this thesis study entitled as "**A PRELIMINARY STUDY ON SPRAY INDUCED GENE SILENCING TECHNOLOGY FOR PROTECTION AGAINST GREY MOLD AND FUSARIUM HEAD BLIGHT DISEASE**" represents my own work which has been done after registration for the degree of MSc at Ege University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications. I also declare that all the information in this document has been obtained and presented in accordance with Ege University academic rules and regulations as well as ethical conduct. Lastly, I declare that, as required by these rules and conduct, I have fully cited and referenced all material and results, which are not original to this work.

21 / 09 / 2021

Reemana FATEMA

ÖZET

GRİ KÜF VE FUSARIUM BAŞAK YANIKLIĞI HASTALIKLARINDAN KORUNMAK İÇİN SPREY KAYNAKLI GEN SUSTURMA TEKNOLOJİSİ ÜZERİNE ÖN ÇALIŞMA

FATEMA, Reemana

Yüksek Lisans Tezi, Tohumluk Bilimi ve Teknolojisi Anabilim Dalı

Danışman: Doç. Dr. Evren KOBAN BAŞTANLAR

İkinci Danışman: Doç. Dr. Ramesh Raju VETUKURI

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RNA interferansı (RNAi), transkripsiyon sonrası gen düzenlemesinde rol oynayan ve çift sarmallı RNAnın rol aldığı doğal bir hücresel savunma sistemidir. Son on yıldaki literatür çalışmalarında, bitki patojenlerine karşı savunma da dahil olmak üzere bir hastalık kontrol stratejisi olarak RNAi'yi kullanmak için teknikler geliştirmeye yönelik çalışmalar bulunmaktadır. Sprey Kaynaklı Gen Susturma (SIGS), tarımda RNAi uygulamasına yönelik kullanılan tekniklerden biridir.

Bu çalışma, hasat edilen mahsullerin besin değerini değiştiren, verim kaybına ve mikotoksin kontaminasyonuna neden olan gri küf ve *Fusarium* baş yanıklığı hastalıklarını kontrol etmek için yöntemler geliştirmek üzere SIGS'yi kullanmayı amaçlamıştır. Bu hastalıklar, ekonomik kayıplara yol açtığı gibi hem insan hem de hayvan sağlığını da tehdit etmektedir.

Çalışmanın amacına ulaşmak için, *Botrytis cinerea*'nın üç geni (*BcCYP51, BcSDHB, BcTUBA*) ve dört *Fusarium graminearum* geni *FgCYP51, FgSDHB, FgBT1, FgBT2*) hedef genler olarak seçilmiştir. Bu hedef genleri tamamlayıcı dsRNA'nın toplu üretimi, dsRNA parçalayıcı enzim olan RNase III enizimi eksik olan bir bakteri sistemi (Escherichia coli) kullanılarak gerçekleştirilmiştir. Çalışmada, dsRNA'nın patojenik büyüme üzerindeki etkisi, uygulamadan sonra gün gün izlenmiştir. En görünür etki, patojenin aşılanmasından yedi gün sonra domates meyvesi (*B. cinerea*), buğday yaprakları (*F. graminearum*) ve buğday başakları (*F. graminearum*) üzerinde gözlemlenmiştir. Test numunelerinde meydana gelen

enfeksiyon, su ve spesifik olmayan dsRNA püskürtmeli kontrol numunelerininki ile karşılaştırılmıştır. *B. cinerea*'nın zayıflatılmış enfeksiyonu, dsRNA (*BcCYP51*, *BcSDHB, BcTUBA*'yı hedefleyen) ile muamele edilmiş domates meyvesinde kaydedilmiştir. Bununla birlikte, *FgCYP51*'i hedefleyen dsRNA, buğdaydan ayrılmış yapraklarda daha az hastalık sergilemiş ve *FgBT1*'i tamamlayan dsRNA, *FgBT*2 buğday başaklarında daha az enfeksiyon göstermiştir. *FgSDHB*'yi hedefleyen dsRNA, kontrollere kıyasla buğday yapraklarında veya sivri uçlarda enfeksiyon açısından herhangi bir önemli farklılık göstermemiştir. Bu ön deneyler, tarımda hastalık kontrolü için SIGS teknolojisinin kullanılmasında umut verici sonuçlar ortaya koymuştur. dsRNA'nın *F. graminearum* üzerindeki kesin etkilerini sonuçlandırmak için daha ileri deneyler gereklidir. Bu ön sonuçlarla, hem sürdürülebilir hem de çevre dostu olan yeni nesil bitki hastalık kontrol yöntemi olarak SIGS, potansiyeli yüksek bir metot olarak öne çıkmaktadır.

Anahtar Kelimeler: SIGS, çift sarmallı RNA, RNAi

ABSTRACT

A PRELIMINARY STUDY ON SPRAY INDUCED GENE SILENCING TECHNOLOGY FOR PROTECTION AGAINST GREY MOLD AND FUSARIUM HEAD BLIGHT DISEASE

FATEMA, Reemana

MSc Thesis, Seed Science and Technology

Supervisor: Assoc. Prof. Dr. Evren Koban BAŞTANLAR

Co-supervisor: Assoc. Prof. Dr. Ramesh Raju VETUKURI

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RNA interference (RNAi) is a natural cellular defense system involving double-stranded RNA, which plays role in post-transcriptional gene regulation. In the last decade, there are studies to develop techniques to employ RNAi as a disease control strategy including defense against plant pathogens. Spray Induced Gene Silencing (SIGS) is one of the techniques for RNAi application in agriculture.

The present study aimed at using SIGS to develop methods to control grey mold and Fusarium head blight diseases, which cause yield loss and mycotoxin contamination, alter the nutritional value of harvested crops. These lead to economic losses as well as threatening of both human and animal health.

To achieve this goal, three genes of *Botrytis cinerea* (*BcCYP51, BcSDHB, BcTUBA*) and four genes of *Fusarium graminearum* (*FgCYP51, FgSDHB, FgBT1, FgBT2*) were selected as target genes. The bulk production of dsRNA complementary to these target genes was carried out by using the bacterial system (*Escherichia coli*) that is deficient in dsRNA degrading enzyme RNase III.

The effect of dsRNA on pathogenic growth was monitored day by day after application. The most visible effect was observed on tomato fruit (*B. cinerea*), wheat leaves (*F. graminearum*) and wheat spikes (*F. graminearum*) seven days post-inoculation of the pathogen. The infection occurred on the test samples were

compared with that of water and non-specific dsRNA sprayed control samples. Attenuated infection of *B. cinerea* was recorded in dsRNA (targeting *BcCYP51*, *BcSDHB, BcTUBA*) treated tomato fruit. However, dsRNA targeting *FgCYP51* exhibited less disease in wheat detached leaves and dsRNA complementary to *FgBT1, FgBT2* showed less infection in wheat spikes. The dsRNA targeting *FgSDHB* did not reveal any significant differences in terms of the infection on either wheat leaves or spikes compared to controls. These preliminary experiments revealed promising results in employing SIGS technology for disease control in agriculture. Further experiments are required to conclude definite effects of dsRNA on *F. graminearum*. With these preliminary results, the potentiality of SIGS can be exploited as a next- generation plant disease control method that is both sustainable and environmentally friendly.

Keywords: SIGS, double-stranded RNA, RNAi

PREFACE

Plant diseases pose a significant threat to world food production exacerbating the challenge in meeting global food requirements. Fungal pathogens reduce agricultural production and pose a threat to human and livestock health by producing mycotoxins and altering the nutritional value of harvested products from crops. The reduction of plant diseases has been carried out by a few paths that harm the environment, human and animal health. Numerous chemical fungicides and physical methods have extensively been used to control plant fungal diseases but several prospects carry the tradeoff making this process inadequate. The development of resistant fungi toward fungicides and the harmful implications of chemical fungicides to the environment has seriously dragged the requirement to implement substitute disease control method considering with sustainable approach.

My thesis focuses on controlling certain plant pathogens infecting major agronomic crops such as wheat, and horticultural crops like tomato by SIGS technology, thus maintaining good production and meeting up enough requirements of food. SIGS efficiency is ensured by its sequence specific mechanism and can be promoted to farmers as Non-GMO biopesticides.

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1. INTRODUCTION

A wide range of fungal diseases causes a loss of 20-25% of harvested crops annually due to fungal infestation and subsequent mycotoxin contamination. *Fusarium* and *Botrytis* species are the most destructive pathogens (Petrasch et al., 2019). The necrotroph *Botrytis cinerea* (Islam and Sherif, 2020) and hemi biotroph *Fusarium graminearum* can transition to necrotroph and survive without living cells (Rampersad, 2020), are difficult to entirely be eradicated thus, forging poor quality of cereal grains and fruits.

1.1 *Botrytis cinerea*

B. cinerea is ranked as the second most destructive pathogenic fungus causing massive yield losses and substantially reducing fruit quality at the post-harvest period (Dean et al., 2012). *B. cinerea* causes grey mold diseases in more than 200 crop species including tomato, kidney bean, cucumber and strawberry by employing different lytic enzymes and phytotoxins in plants to acquire nutrients from the host (Espino et al., 2014; Islam and Sherif, 2020). The pathogen stimulates fruit decaying at the post-harvest period that accounts for 15-50% of yield losses making challenging to commercialize the horticultural fruits (Bu et al., 2021). During storage, the decayed fruits produce several toxins which may have carcinogenic effects posing harm to human and animal health.

Botrytis is saprophytic fungi that remain dormant in unfavourable conditions making it a more vulnerable pathogen to be controlled. *B. cinerea* produces conidia at both sexual and asexual stages and chlamydospores at unfavourable conditions (Romanazzi and Feliziani, 2014). Conidia of *B. cinerea* is dispersed by air or water and penetrates new host plants through natural openings from an infected plant. High humidity with 25°C temperature is beneficial for *Botrytis* sporulation which starts to rot leaves, flowers and fruits (Iqbal et al., 2021). *Botrytis* can grow at 0°C, thus having the capacity to infect fruits and vegetables in cold storage (Romanazzi and Feliziani, 2014).

1.1.1 Existing approaches to control *Botrytis* pathogen

There are several approaches to control *B. cinerea* comprising physical, chemical and biocontrol treatments (Romanazzi et al., 2016). Physical treatments include UV, low temperature, thermal radiation (Collemare et al., 2019; Bu et al., 2021). Chemical additives such as sodium benzoate and potassium sorbate can be applied to fruits before or after harvest can also prevent fruit rotting (Amirpour et al., 2015; Din et al., 2019; Kaur et al., 2019; Youssef and Hussien, 2020). Application of fungicides (Pyridine-carboxamides, Pyridinyl-ethyl-benzamide, Pyraclostrobin, Hydroxyanilides) secures 40% pre and post-harvest crops losses due to *B. cinerea* (Pedras et al., 2011; Romanazzi and Feliziani, 2014). However, the tradeoff is that *Botrytis* strains resistant to fungicides belonging to the quinone outside inhibitor (QoI) class have already emerged (Rupp et al., 2017). Resistant strains have been reported in grape (Panebianco et al., 2015), strawberry (Amiri et al., 2013), tomato (Konstantinou et al., 2015) and raspberry (Weber, 2011). Biocontrol treatments are engaging usage of antagonism effect of *Bacillus*, *Pseudomonas syringae*, *Cryptococcus albidus*, *Candida sake* (Jiang et al., 2018; Romanazzi and Feliziani, 2014; Chen et al., 2020). However, each of the strategies depends on the commodity and most of the time they are complemented with other time-consuming strategies and yet not entirely effective. Hence it is necessary to develop molecular schemes to have effective treatments to control *Botrytis* (Romanazzi and Feliziani, 2014).

1.2 *Fusarium graminearum*

F. graminearum is the fourth most destructive fungal pathogen in the filamentous pathogenic fungal genera, reduces the quality of cereal grains and yield (Dean et al., 2012). Over decades, *F. graminearum* has surged concerns due to its compelling saprophytic nature and resultant accumulated toxins in grains. *F. graminearum* infestation is reckoned as the significant problem of wheat. Since wheat is considered as the second most important crop in terms of food security providing the staple nutrients for 40% of the world population (Giraldo et al., 2019), diseases caused by *F. graminearum* are serious concern to be attenuated.

F. graminearum commences infection by spores on grain tissue surfaces of wheat and barley. The hemi-biotrophic fungus *F. graminearum* produces sexual, asexual and chlamydospore. The sexual ascospore gets released by the activity of perithecia (Geiser et al., 2013) and the asexual conidia get dispersed by wind or rain (Trail et al., 2005). Anthesis is when the spores usually the find the gateway to enter the cereal plant body causing the head blight infection in the spikes (Hooker et al., 2002). It has also been observed that late infection can also cause a significant accumulation of mycotoxins in barley and oats kernels (Yoshida et al., 2007; Tekle et al., 2012;) . The spores gradually develop into hyphae penetrating the tissue of lemma and palea of grains spreading through vascular tissues of spikelet (X. W. Zhang et al., 2013). Eventually, the fungus shrivels and contaminates the spikes with mycotoxins (deoxynivalenol, nivalenol and zearalenones) giving rise to the Fusarium head blight (FHB) in cereal crops. *F. graminearum* can also cause wilting, seedling blights, foot rot, root rot and cankers causing significant yield loss in major cereal crops i.e. wheat, barley, rice and oats (Leplat et al., 2013).

Spores of *F. graminearum* can travel several kilometers away infecting plants thus facilitating the epidemic process. *F. graminearum* acts as a saprophytic pathogen, being able to survive on contaminated crop residue, which plays essential role of source for the pathogen inoculum. Epidemics can emerge from diseases risen from *F. graminearum* because of contaminated grains that are toxic to both human and animal health. *F. graminearum* spores can potentially grow throughout the whole growing season (Hellin et al., 2018). A study conducted on Fusarium inoculated wheat and maize field has reported the phenomenon of growing perithecia and ascospore during the whole season (Karlsson et al., 2021). Proper agronomic practices, tillage, crop rotation might reduce the probability of infestation but still there is requirement to have a control strategy after *Fusarium* infection (Karlsson et al., 2021).

1.2.1 Existing approaches for controlling *Fusarium* pathogens

Developing genetically resistant crop variety is one of the best prevailing measures that are extensively used. However, developing resistant cultivars through conventional breeding is an enormous challenge and a prolonged process (Cheng et

al., 2015). Besides, chemical fungicides such as Demethylation Inhibitors (DMI) have extensively been used to control *F. graminearum*. DMI inhibits ergosterol biosynthesis of *Fusarium*, distorting fungal membrane integrity (Aline Koch et al., 2016). However, the re- evolving *Fusarium* manifests resistance against fungicides (Höfle et al., 2020). Moreover, the harmful implications of chemical fungicides to the environment have seriously pulled up the requirement to implement alternative disease control methods embellished with a sustainable approach.

Several agronomic practices are prevailing to control infection of *Botrytis* and *Fusarium* including crop rotation and irrigation management in horticultural and cereal crops. However, these practices are influenced by the environment keeping a gap from acquiring definite success in crop protection from fungal diseases (Qi et al., 2019a). Given the situation, exploitation of biomolecules such as nucleic acid, proteins, lipids, sugars which would engage naturally occurring pathways controlling plant disease can be an enormous change to the crop protection arena in a sustainable way. RNA interference-based disease control method can be an emerging novel technology involving plant inherent defense mechanism that can be more effective and sidestepping the hazardous chemical control against fungal crop diseases (Qiao et al., 2021).

1.3 Mechanism of RNA Interference

RNA interference (RNAi) is a conserved cellular defense mechanism of eukaryotes against foreign molecules mediated by small RNAs (sRNA) prompting post-transcriptional gene silencing (PTGS). RNAi is a phenomenon that can be exploited in fundamental research as well as implemented on field level crop protection. Utilizing RNA biopesticides are produced based on RNAi mechanism and sprayed into plants inducing gene silencing of virulent genes of pathogens. Thus, spray induced gene silencing (SIGS) has become a hot topic in the context of controlling plant diseases (Aline Koch et al., 2016; Kalyandurg et al. 2021; Vetukuri et al. 2021).

The silencing of gene primarily procured with cleavage of precursor double-stranded RNA (dsRNA) into non-coding, short 20-24 nucleotide (nt) small

interfering RNA (siRNA) which is performed by RNase III-like enzyme called Dicer (Ketting, 2011). The resulting siRNAs get loaded with RNase protein Argonaute forming RNA induced silencing complex (RISC). The activated RISC unwinds the double-stranded siRNAs and chops into single-stranded RNA (sRNA) in an ATP dependent reaction (Meister, 2013). The generated sRNAs are antisense to target mRNA sequence (Borges and Martienssen, 2015) which eventually binds to target sequence impeding/repressing translation of target transcript or decaying mRNA resulting in gene silencing (Chang et al., 2012) (Figure 1.1).

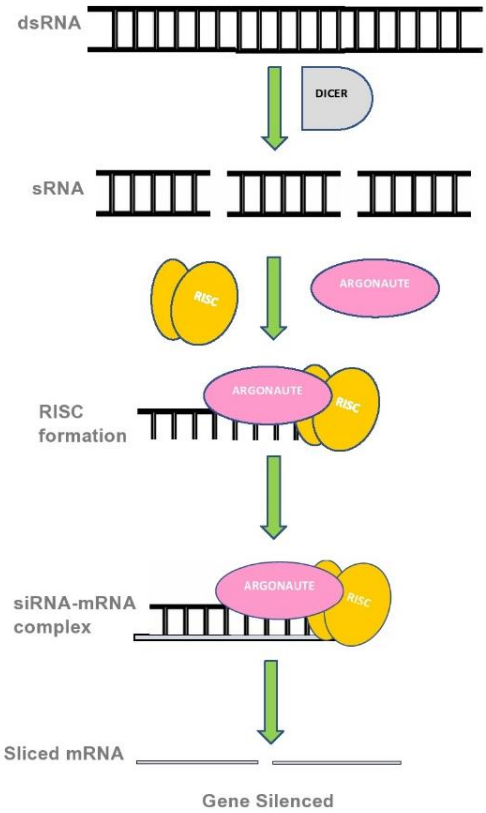

Figure 1.1. Mechanism of RNAi in gene silencing

RNAi was first discovered by overexpressing the Chalcone gene (CHS) gene in violet petunia which unexpectedly produced 42 percent white petunia as a result of silencing biosynthesis of anthocyanin (Napoli et al., 1990). *Neurospora crassa* was first described as model fungi for RNAi pathway study and its major component of RNAi mechanism is comprised of Dicer, Argonaute, and RNA-dependent RNA-polymerases (RdRps) (Liu et al., 2010). Pathways involved in RNAi have diversification depending on their function that is involved in gene

silencing. For instance, in Arabidopsis it has been reported that Dicer-like protein (DCL) has four homologs (DCL1, DCL2, DCL3, and DCL4) (Voinnet, 2009; Dalakouras et al., 2020) and each of the homologs are differed by its function to synthesize different sizes (18, 22, 24, 21 nucleotides respectively) of short interfering RNA (siRNA). siRNA of 21 nucleotides (nt) are known to be loaded with Argonaute 1 (AGO1) leading to post- transcriptional gene silencing whereas, siRNA 22-nt incorporated with AGO1 employ RDR6 which proceeds in synthesizing secondary siRNA. In case of 24-nt siRNA, DNA methylation is activated by involving with AGO4 and DNA methyltransferase (Chan et al., 2004). *F. graminearum* RNAi machinery is composed of two Dicer proteins (FgDicer1 and FgDicer2), two ARGONAUTE proteins (FgAgo1 and FgAgo2), and five RNA-dependent RNA polymerases (RdRps) (FgRdRp1–5) (Chen et al., 2015).

1.4 Objective

Modern agriculture focuses on combining the existing fungal disease control methods taking grip with sustainable and eco- friendly approaches. Thus, the present study aims to evaluate the prospects of RNAi based Spray Induced Gene Silencing (SIGS) method to control Grey mold disease on tomatos and Fusarium head blight (FHB) in wheat. To obtain dsRNA to prevent grey mold and FHB diseases, pathogenic genes involved in the mycelium or spore production of *B. cinerea* and *F. graminearum* were targeted. Thus, dsRNA targeting four pathogenic genes (*FgCyp51, FgsdhB, FgBt1, FgBt2*) of *Fusarium* and three pathogenic genes of *Botrytis* (*BcCyp51, BcsdhB, BcTubA*) involved in the fungal infection was produced. The production of dsRNA was carried out by *Escherichia coli* bacterial system. Target dsRNAs were ligated with L4440 vectors and the recombinant vector was transformed into HT115 strain of *E. coli* and the bacteria was cultured. Subsequently, the dsRNA was extracted from bacterial culture and sprayed into plant leaves (wheat), spike (wheat) and fruit (tomato) and the infection was observed in Stereomicroscope and Biorad. The objective of the study is to implement the SIGS method characterized by its sequence specificity and possessing no residual effect in fungal disease control. SIGS can be tagged as a sustainable approach ensuring food security without compromising the natural genetic construction of crops.

2. REVIEW OF LITERATURE

2.1 Host Induced Gene Silencing vs Spray Induced Gene Silencing

RNAi phenomenon has been exploited in the plant protection arena silencing fungal pathogenic genes. The delivery of dsRNA can be either by producing transgenic plants or topical application of dsRNA on plants. Genetically modified plants expressing sRNAs or dsRNAs silencing pathogen virulence- related genes are known as Host Induced Gene Silencing (HIGS) (Cai et al., 2019). HIGS is based on an inverted repeat sequence which is a homolog to a gene of virulent pathogen and the sequence is integrated to plant genome by genetic transformation to generate sRNA or dsRNA; eventually, the fungal pathogen uptake the sRNA or dsRNA silencing pathogenic gene (Qi et al., 2019b). HIGS can be an emerging novel technique to control plant fungal diseases which has also been demonstrated in several studies (A Koch et al., 2020; Nowara et al., 2010; Qi et al., 2018; Song et al., 2018). It can be exploited to crops that are in high value dropping yield and post-harvest losses.

Nevertheless, implementation of HIGS is restricted due to the unavailability of transformation protocols for different vegetable, grain crops or fruit trees (Capriotti et al., 2020; Aline Koch and Kogel, 2014). Besides, the consequences of genetically modified plants are unknown and ban on GMO techniques in Europe for food and feed production (Kleter et al., 2018; Tsatsakis et al., 2017) may raise more concerns among the public to accept the HIGS technology to control the crop disease. Thus, there is a compulsion on plant protection research to figure out more sustainable and environmentally friendly approaches to control devastating fungal infection caused by *Botrytis* and *Fusarium*.

Given the facts of HIGS restrictions, topical application of dsRNA can an alternative method to manoeuver RNAi based disease control method. Equivalent to HIGS, Spray Induced Gene Silencing (SIGS) has also been performed in the alleviation of infection in hosts (Wang et al., 2016) Pathogenic genes of *Botrytis* and *Fusarium* have been silenced by sRNAs processed from environmental dsRNA applied by SIGS method (Aline Koch et al., 2016; Wang et al., 2016). SIGS

experiments were conducted by spraying dsRNA targeting cytochrome P450 which plays a crucial role in ergosterol biosynthesis of *F. graminearum* into barley leaves which eventually inhibited of fungal growth on leaves (Aline Koch et al., 2016). The study showed that spraying detached barley leaves with 791-nt long CYP3-dsRNA, designed as complementary sequences to *CYP51B, CYP51A,* and *CYP51C* prior to fungal infection showed inhibition of disease and reduced lesion. The resistance against *Fusarium* was also found in adjacent non-sprayed leaves. The suppression of disease was supported by the measurement of quantitative PCR which showed less *CYP51* gene expression in dsRNA sprayed leaves.

A similar investigation (Wang et al., 2016) associated with *Botrytis* was carried out to control grey mold disease by spraying sRNAs targeting *BcDCL1* and *BcDCL2* on fruits of tomato, lettuce, strawberry; rose petals and Arabidopsis leaves observing resistance toward disease. They also observed efficient reduced infection of *Botrytis* in double mutants *dcl1/dcl2* on those crops, affirming the efficiency of SIGS.

Along with efficiency, the effective duration of dsRNA is crucial for being considered as a disease control agent. Koch *et al.* (2016) also demonstrated the duration of dsRNA effectiveness with northern blot analysis and convincing the fact that dsRNA was stable up to seven days on the surface of dsRNA sprayed leaves. Agreeing with the point, Wang *et al*. (2016) also proved that fruits and vegetables can be protected up to 8 days from grey mold disease by dsRNAs. To perceive SIGS efficiency, spraying dsRNA targeting *DCL1* and *DCL2* gene of *Botrytis cinerea* experiments were executed on strawberries (Sabbadini et al., 2021).

Uptake efficiency of dsRNA by fungus was recently investigated (Qiao et al., 2021) and demonstrated that dsRNA was actively up taken with divergent levels of efficiencies by several fungal pathogens e.g. *Rhizoctonia solani, Sclerotinia sclerotiorum, Verticillium dahlia, Aspergillus niger* and vaguely taken by beneficial fungus *Trichoderma virens*.

Plants are protected from pathogen infection by several layers of defense mechanism comprising microbe and pathogen- associated molecular pattern

(MAMP) and (PAMP) triggered immunity (PTI) and effector triggered immunity (ETI) (J. Zhang et al., 2010). Pathogens deliver effectors to plants in order to facilitate their virulence in the host inhibiting the plant immunity. Topically applied sRNA induces plant innate defense mechanism acting as MAMP and causing PTI in Arabidopsis (Lee et al., 2016). It has been found that *Botrytis* produces siRNA effectors to suppressing the host RNAi system, thus progressing in infection of plants (Weiberg et al., 2013). The suppression of the host immune system is characterized by programmed cell death leading to asymptomatic colonization of *B. cinerea* before the necrotrophic phase of infection. *B. cinerea* RNAi machinery constitutes of DCL1 and DCL2 proteins that hijack the host defense system by producing different siRNAs and delivering them into the host to bind with AGO1 of host RNAi machinery. A study was done profiling *Botrytis* sRNA library on *Botrytis* infected plant tissue finding that Bc-siR3.2, Bc-siR5, and Bc-siR3.1 can aim at several defense factors of host plants (Weiberg et al., 2013). The siRNAs of *B. cinerea* can target the genes that are involved in maintaining cell wall integrity (cell wall associated kinase e.g. WAK) (Brutus et al., 2010; Ferrari et al., 2013), hormonal signaling in defense pathway (mitogen- activated protein kinase e.g. MPK1, MPK2, and MAPKKK4) (Mandadi and Scholthof, 2013) or cell homeostasis (Peroxiredoxin e.g. PRXIIF) (Finiti et al., 2014; Islam et al., 2017).

2.2 Different Methods of Producing dsRNA

There have been diverged studies conducted to find out potential candidate genes of the fungal pathogen to be silenced by HIGS and SIGS turning down fungal infection on plants (Gebremichael et al., 2021). Synthesis of exogenous dsRNA is carried out both *in vitro* and *in vivo.* Both conditions produced the dsRNA that attenuated the fungal growth or altered fungal morphology developing reduced fungal disease symptoms. Enzymatic transcription and chemical synthesis are embodied *in vitro* methods. Enzymatic transcription method for dsRNA synthesis is based phenomenon of annealing of the two single strands of RNA (ssRNA) using ex pensive kits.

On the other hand, this expensive method is not reasonable to produce a bulk amount of dsRNA to for a field trial. Unlike Enzymatic transcription method, the

chemical synthesize of dsRNA is suited for dsRNA bulk production. Nevertheless, the expensive cost is a drawback of *in vitro* method (Gebremichael et al., 2021). *In vivo* methods engage genetically engineered prokaryotic organisms like bacteria (*Escherichia* coli, *Pseudomonas syringae*) and yeast (*Yarrowia lipolytica*) to produce large quantities of dsRNA(Voloudakis et al., 2015; Álvarez-Sánchez et al., 2018). Bacterial based dsRNA production has already been established in many studies (Lisa et al., 2001; Newmark et al., 2003; Ahn et al., 2019; Z. Chen et al., 2019a; Niño-Sánchez et al., 2021) and found to yield a colossal amount that can be applied to crops in field- level experiments controlling disease at low cost. RNase III (RNA degradation enzyme) deficient *E. coli* strain HT115 (DE3) having recombinant plasmid L440 containing two inverted T7 promoters is generally used for dsRNA production (Ahn et al., 2019). The T7 promoter mediated transcription is induced by isopropyl β-d-1- thiogalactopyranoside (IPTG) finally activating dsRNA production by the bacterial system (Ahn et al., 2019). Later the dsRNA from the bacterial system is isolated with several methods such as sonication or heat treatment.

Cost concerning dsRNA production from fungus sequence derived in *E. coli* bacteria has been calculated around 1 USD per 1g (Tenllado et al., 2003). Along with low cost, easy handling, a rapid growth rate of bacteria containing dsRNA make synthesizing bacterially expressed dsRNA is enlisted as a sustainable tool alternative to chemical control of plant insects and diseases (Terpe, 2006). It is also been postulated that depending on the pathogen and host plants, delivery techniques, target sensitivity toward dsRNA, 2 to 10 grams of dsRNA is required for 1 hectare of land (Das and Sherif, 2020). Although the presence of bacterial homologous DNA molecules and the residual effect of lysate bacteria might affect the quality of dsRNA quality, yet this process is constructively applied against fungus, insects, viruses and worms (Gebremichael et al., 2021).

2.3 Factors Influencing Efficiency of Exogenous dsRNA Application

The success of topically applied dsRNA to control fungal disease critically depends on plants capacity to absorb exogenously applied dsRNA. The dsRNA absorbing capacity of both plants and fungi is subject to different application

methods, size and length and concentration of dsRNA. Both plants and fungi can uptake dsRNA from environment or the dsRNA trafficking can prevail between the cross-kingdom (plant-fungus) (Wytinck et al., 2020). Although it has been speculated that sRNAs can move bi- directionally (Wang et al., 2016) between plant and fungi, yet the transport mechanism of sRNAs or dsRNAs from plants to fungi has not been investigated in detail. However, several studies have built a concept that sRNAs or dsRNAs can move by symplast or apoplast in form of naked or encased in extracellular vesicles (Kehr and Buhtz, 2008; Aline Koch et al., 2016). dsRNA can move through plants via vascular bundle of plants and silence fungi on dsRNA non-treated plant parts. The fact was confirmed in a study that the dsRNAs moved intact from dsRNA treated local area tissues to dsRNA non-treated distal area tissues by the plant vascular system (Nerva et al., 2020).

2.3.1 Uptake capacity

The efficiency of silencing of the fungal gene also depends on the dsRNA absorbing capacity of fungus from dsRNA sprayed plants and delivering methods of dsRNA influences the efficiency. Wang *et al*. (2016) showed that *Botrytis* spores germinating on Agar media have up taken fluorescein-labelled dsRNA. They also co- incubated fluorescein-labelled dsRNA with isolated protoplast culture of *Botrytis* liquid culture and observed similar results after 12 hours and 20 hours post dsRNA application. The study correlated the uptake of dsRNA with virulence inhibition of *Botrytis* and silencing of RNAi machinery genes e.g. *DCL1* and *DCL2*. Similar observations were also exhibited in case of *F. graminearum* when dsRNA targeting ergosterol biosynthesis genes (*CYP51A, CYP51B, CYP51C*) was incubated in fungal conidia and resulted silencing the genes after 24 hours (Aline Koch et al., 2016). Similar studies are also found in other pathogens such as *Sclerotinia sclerotiorum, Magnaporthe oryzae* and *Colletotrichum truncatum* (McLoughlin et al., 2018; Song et al., 2018; Gu et al., 2019)

2.3.2 Different delivery methods of dsRNA

The potentiality of absorbing dsRNA by plants against *Botrytis* was displayed in grape fruit, leaf and petiole in natural environment instead of *in vitro* conditions

(Nerva et al., 2020). Three different dsRNA delivery methods (high- pressure spraying, petiole adsorption, high pressure nebulization) were also examined to control post-harvest disease caused by *Botrytis* on grape bunches, among them spraying dsRNA with high- pressure was proven most effective method. Besides, there are also several delivery methods existing for dsRNA to plant e.g. injection into plants, soil application, application by brush, root soaking, injection, post-harvest spraying, infiltration, soil drench (Whangbo and Hunter, 2008; Li et al., 2015; Wang et al., 2016; Dalakouras et al., 2018, 2020b). Conceding the success of spraying dsRNA at high pressure was also reported in another study where green fluorescent protein (GFP) transgene was silenced locally and systematically in *Nicotiana benthamiana* (Dalakouras et al., 2016) in comparison to other techniques such as gene gun, infiltration etc. On the contrary, a study has been found where the exogenous dsRNA was applied by spreading the dsRNA on transgenic *Arabidopsis* adult plants without any agent or techniques and efficient silencing of transgenes e.g. green fluorescent protein (*EGFP*) and neomycin phosphotransferase–II (*NPTII*) transgenes were observed (Dubrovina et al., 2019). The study also speculates on the significance of different concentrations of dsRNA (0.1, 0.35 and 1.0 µg/µl) and they have found 0.35 µg/µl to be the optimum concentration for silencing the transgenes *EGFP* and *NPTII.*

2.3.3 Length of dsRNA

The effects of dsRNA- mediated gene silencing also differ with the various dsRNA lengths designed to target virulent genes. Effectiveness of different lengths of dsRNA (315, 596, and 977-bp) targeting viral genes was experimented in *Nicotiana tabacum* leaves implying that shorter length of dsRNA exhibited efficient knocking down of viral genes (Tenllado and Díaz-Ruíz, 2001). Conversely, longer dsRNA (more than 60bp) was fed in diet of larvae of Western Corn Rootworm which exhibited a high mortality rate than the diet containing shorter dsRNA (Bolognesi et al., 2012)**.**

2.4 Safety of RNAi Technology in Plant and Human Health

Controlling plant disease by RNA interference is receiving much popularity because it is environmentally friendly which is considered as green technology. Few studies have proved the fact that either by SIGS or HIGS, no harmful effect is recorded in plants or humans or pollinators (Bachman et al., 2016; Tan et al., 2016). Sequence- specific dsRNA can be constructed by identifying the pathogenic genes responsible for fungal infection and targeting those pathogenic genes. Thus, avoiding off targets that might be crucial to avoid unexpected gene silencing in the host or human or pollinators (Mcloughlin et al., 2018).

Bachman *et al*. (2016) experimented effect of dsRNA in physiology, reproduction and nutrient assimilation capacity of several invertebrates (*Apis mellifera, Coleomegilla maculate, Eisenia adrei*) and vertebrates (*Ictalurus punctatus, Gallus domesticus*) and soil microbes. The authors did not find any significant changes in the organisms due to dsRNA application irrespective of the sources of dsRNA. They also inferred that dsRNA targeting corn root worm did not interfere normal behavior or distribution of honey bees. In another study (Tan et al., 2016), the growth and longevity of honey bees were investigated after feeding dsRNA to the larva and adult of honeybee at 10 times higher doses than usual dose of dsRNA that is exposed in environment. dsRNA was designed to target *Snf7*, an ortholog of *DvSnf7* of western corn rootworm, and after feeding the growth and longevity remained unchanged of the honeybee. Studies (Christensen et al., 2013; Petrick et al., 2015) also reported the lack of efficacy of dietary sRNA in mammals by not showing any destructive cellular effect in the gastrointestinal tract or bloodstream after consuming sRNA or dsRNA targeting its vital ATPase. Notably, human has long tracks of dietary consumption of dsRNA by consuming virus-infected food showing no adverse effect in health probably due to dsRNA nuclease degradation by enzymatic reaction in human stomach and guts (Jensen et al., 2013)

The stability of dsRNA is another point that is considered as core advantage of RNAi techniques. Double- stranded RNA is thermodynamically more stable than single- stranded RNA which gives in more resistance toward degradation by nuclease activity (Nicholson, 2014; Wang and Jin, 2017). The stability of actin-

dsRNA against Colorado potato beetle was proven to be stable on leaves for 28 days after foliar application of dsRNA in greenhouse conditions (San Miguel and Scott, 2016). On the other hand, dsRNA lacks stability and degrades once it enters the soil and aquatic system within 24- 96 hours due to the presence of nuclease activities that ensures no residual effect of dsRNA (Dubelman et al., 2014; Fischer et al., 2016; Albright et al., 2017).

Besides, food products (oil producing crops, cereals) undergo many processes such as thermal treatment, solvent purification, baking, fermentation, microwaving, acidification, alkalization, destabilizing and degrading any kind of nucleic acid before consumption (Mcloughlin et al., 2018).

2.5 Limitations

Harnessing the technique of SIGS has seized attention for its sequence specificity and possessing the potentiality of being part of sustainable plant disease control. Nevertheless, SIGS is bound to certain limitations that are yet to be resolved. Firstly, the stability of dsRNA on plants has come into a query which is found to be stable up to 5days by bacterially (*E. coli*) expressed dsRNA in plants whereas, dsRNA encapsulated in Nano clay particles have been exhibited stability up to 20 days after spray (Tenllado et al., 2003; Mitter et al., 2017). Although the tradeoff is that Nano clay sheets are expensive and the effects of such carriers in human health are unknown, thus pivoting future investigation on increasing stability of dsRNA. Moreover, the stability of dsRNA in field- level application is still matter of concern.

Secondly, UV radiation, water/soil, microbe produced ribonucleases present in the environment can degrade dsRNA (Dubelman et al., 2014). Till now S1 extracellular nuclease of fungus has been found to degrade single- stranded DNA (ssDNA), RNA and dsRNA although in case of dsRNA targeting insects, several nucleases (Eri-1, Dicers and dsRNases) has been found in the guts of insects, hence evolving resistant insects (Peng et al., 2018). Resistant strain corn rootworm (*D. virgifera*) has already been observed by confirming by less uptake of dsRNA by insects. The insects developed 130-fold higher resistance against dsRNA uptake

due to single mutation of recessive gene. Although the mutation of resistant strain of insect was executed in laboratory pointing to the probability of such resistance in nature (Khajuria et al., 2018).

Moreover, the RNAi mechanism works well for those pathogens which own RNA machinery within its system. Thus, dsRNA uptake depends on the fungal RNAi mechanism. Corn smut causal agent *Ustilago maydis* lacks RNAi machinery making it unable to process dsRNA to produce siRNAs to silence its pathogenicity genes, thus, contributing as a poor candidate for RNAi technology for disease control method (Billmyre et al., 2013). The pathogens can also appear with new genetic combinations hence, pre-designed dsRNA molecules might not work (Rampersad, 2020). In addition, studies regarding RNA stabilizing agents, factors influencing RNA uptake or mode of translocation of dsRNA or sRNA from plants to pathogen are yet not much well deciphered (Dubrovina and Kiselev, 2019).

RNAi technology is sustainable, environmentally friendly solution that retain the potentiality for combating with plant diseases. It is considered as next-generation control strategy to protect plants ensuring food security for global food production. Although several aspects are still required to investigate, RNAi technology can still be reckoned as a sustainable approach dealing with food cultivation challenges.

3. MATERIALS AND METHODS

3.1 Primers For Targeted Genes of *Fusarium* and *Botrytis*

Considering the fungicide role of action, seven genes were targeted among which three genes belong to *Botrytis* (*BcCyp51*, *BcsdhB*, *BcTubA*) and four genes belong to *Fusarium* (*FgCyp51*, *FgsdhB*, *Fgbt1*, *Fgbt2*) (Table 3.1). Primer pairs were designed on the non-conserved region using 'Snapgene', in a way that the PCR may overlap ensuring the presence of all selected PCR amplicons keeping the gene size ranging within 249-493 bp (Supplementary Tables 1 and 2 given in the Appendix).

Table 3.1. Gene name and their function

No	Gene	Function	Reference
1	*BcCyp51*, *FgCyp51*	Sterol biosynthesis, ascospore production	(Fan et al., 2013; C. Zhang et al., 2020)
2	*BcsdhB*, *FgsdhB*	Succinate dehydrogenase inhibitor, disrupts mitochondrial respiration	(Dubos et al., 2013; Samaras et al., 2016)
3	*BcTubA*	A major component of microtubule	Database of Uniprot (https://www.uniprot.org/uniprot/P53373) (S. Liu et al., 2013)
4	*FgBt1*, *FgBt2*	Assemble microtubules and involved in hyphal growth	(S. Liu et al., 2013; Zhao et al., 2014)

3.2 DNA Extraction of *B. cinerea* and *F. graminearum*

DNA was extracted from both pathogens using DNeasy Plant Mini Kit. *Fusarium* and *Botrytis* were collected from solid V8 media and were cultured in liquid V8 media and collected into 2ml of centrifuge tubes. Each sample weighed <100mg and was disrupted in liquid nitrogen by mortar and pestle. While there was no tissue clump observed, 400 µl Buffer AP1 and 4 µl RNase were added to the

samples and were collected into new centrifuges tubes and incubated for 10 min at 65°C. On the next step, 130 µl Buffer P3 was added to the lysate and incubated on ice for 5 minutes to precipitate the cell proteins and polysaccharides. After the incubation the lysate of two fungi was centrifuged for 5 min at 20.000g. The lysate was pipetted to the QIAshredder Mini spin column provided with the kit and the centrifugation was repeated again for 2minutes. The flow- through was transferred into new tubes without disturbing the cell debris and 1.5 volumes of Buffer AW1 was added to the lysate. The lysates were collected to the DNeasy Mini spin column for centrifugation and the resulting flow- through was discarded. After the step, 500 µl Buffer AW2 was repeatedly added to the samples 2 times so that the membrane of the column gets dry and the flow through gets separated from the DNA. Finally, the samples were eluted with 30 µl of Elution buffer and incubated at room temperature, collected into 1.5ml centrifuge tubes. Nanodrop measurement showed that the concentration was 0.39 ng/ µl and 1.675 ng/µl for *Fusarium* and *Botrytis*.

3.3 PCR and PCR Product Purification

PCR was carried out for extracted DNA from *Botrytis* and *Fusarium*. The reaction mix was containing 5X H5 buffer (10 µl /sample), 10mM dNTPs (1 µl /sample), Phusion polymerase (0.5 µl / sample), forward primer (2.5 µl for each gene sample), reverse primer (2.5 µl for each gene sample), DNA template of 10 ng for each gene sample and distilled water making volume up to 50 µl. The PCR was confirmed by running samples in 1% agarose gel (1g Bacto Agar dissolved 100 ml of 1x TAE buffer). After confirmation from gel, PCR products were purified using the 'QIA quick PCR Purification' kit and the quantity was estimated using Nanodrop spectrophotometer.

3.4 Plasmid Extraction and Purification

Plasmid L4440 (Addgene plasmid # 1654; http://n2t.net/addgene:1654; RRID: Addgene_1654) containing bacterial culture was kept in shaking incubator at 37°C for overnight. The next day the bacterial culture was extracted using Thermo Scientific™ GeneJET Plasmid Miniprep Kit following User Manual's instuctions. First, the bacterial culture was centrifuged at 8000rpm for 2 minutes and the remaining pellet was resuspended in 250 µl of resuspension buffer, 250 µl of lysis buffer immediately followed by 350 µl of Neutralization solution. The

solution was centrifuged and the supernatant was passed through columns and washed by wash solution by twice repeated steps. Later, 50 µl of elution buffer was added to columns kept in new nuclease- free Eppendorf tubes and centrifuged. The extracted plasmids were collected and stored into -20°C.

Later, 20 µl of the extracted plasmids were run through 1% of agarose gel (1g Bacto Agar dissolved 100 ml of 1x TAE buffer) and the DNA was excised and collected into 2ml Eppendorf tubes. The plasmids were purified using Zymoclean Gel DNA Recovery Kit following User Manual's instuctions. The weight of the plasmid containing gels were measured and 3 volumes of ADB buffer was added to each volume of agarose excised in the tubes. The samples were incubated at 50°C for 10 minutes and vortexed. Subsequently, 200 µl of DNA Wash Buffer was added and centrifuged and the flow through was discarded. Later the samples were eluted with 10µl of elution buffer and were collected in nuclease- free 1.5ml Eppendorf tubes.

3.5 Restriction Digestion of PCR Products and Plasmid L4440

Plasmid L4440 and PCR amplified fragments of all the seven genes (*BcCyp51, BcsdhB, BcTubA, FgCyp51, FgsdhB, FgBt1, FgBt2*) were digested with their respective restriction enzymes (1 unit of enzyme for each) (Table 3.2). One µg of the samples (plasmid and genes) were digested with 1µl of each restriction enzyme, followed by incubating the samples for 15-30 minutes at 37°C. The plasmids were run into 1% agarose gel (1g Bacto Agar dissolved 100 ml of 1x TAE buffer) and the desired restricted plasmid fragment were purified from a gel.

Table 3.2. List of restriction digestion enzymes for each plasmid and PCR product

No.	Template	Restriction Enzyme 1	Restriction Enzyme 2
1.	L4440	*Hind*III	*Spe*I
2.	L4440	*Hind*III	*Sma*I
3.	L4440	*Xho*I	*Sac*I
4.	*FgCyp51*	*Hind*III	*Spe*I
5.	*FgsdhB*	*Hind*III	*Sma*I
6.	*FgBt1*	*Hind*III	*Sma*I
7.	*FgBt2*	*Hind*III	*Sma*I
8.	*BcCyp51*	*Sac*I	*Xho*I

9.	*BcsdhB*	*Hind*III	*Spe*I
10.	*BcTubA*	*Hind*III	*Sma*I

3.6 T4 Ligation

Ligation mixture included 4 µl of 5x rapid ligation buffer, 50ng of vector DNA, insert DNA (vector: insert = 3:1), T4 Ligase 1 µl and nuclease- free water to make volume up to 20 µl for each sample of digested PCR products following the protocol provided by the website Addgene.org (https://www.addgene.org/protocols/dna-ligation/). The mixtures were pipetted to mix thoroughly and incubated for ten minutes at room temperature.

3.7 Transformation into HT115 (DE3)

Transformation was performed into *E. coli* vector and the competence cells (DE3 strain) (https://cgc.umn.edu/strain/HT115(DE3)) were taken out of -80°C and thawed on ice. The ligated products (20 µl) of DNA were taken into 50µl of competence cells into microcentrifuge tubes and incubated in ice for 30 minutes. Following the incubation, the cells were given heat shock (42°C) for 60 seconds and sample were back on the ice for 2 minutes. Later, 300µl LB Broth (without agar and antibiotics) were added to the bacteria and kept in a 37°C shaking incubator for one hour. The transformed cells were plated with L shaped streaks into a 10 cm LB agar (20ml per plate) plate with antibiotics Ampicillin (100µg/ml) and Tetracycline (6µg/ml) 37°C for overnight.

3.8 Colony PCR

Four random colonies were picked from each sample of transformed petri plates and were collected in Luria Broth (LB) (25gm/l LB high salt, 15g/l Bacto Agar- EC Number:232-658-1) supplemented with Ampicillin (100µg/ml) and Tetracycline (6µg/ml) antibiotics. Subsequently, the cultures were kept in a 37°C shaking incubator overnight to grow. Same colonies (four) per sample from the petri plates were collected in PCR tubes containing 50µl of distilled water. For colony PCR, 2 µl of each sample from that PCR tubes was taken for a 20ul PCR reaction

(denaturing temperature 94°C, annealing temperature 55°C, extension temperature 72°C) following same procedure mentioned above section 3.3 PCR and PCR product purification. The rest of the culture (48µl) of the sample from that PCR tubes were set up for culture overnight at 37°C. Four replicates were prepared for each sample.

3.9 Bacterial Culture and dsRNA Induction

Optical density (OD_{600}) of overnight grown bacterial culture at 37°C containing transformed plasmids was measured in a spectrophotometer. Each culture was adjusted to $OD_{600} \approx 0.2$ with freshly prepared LB broth containing antibiotics (ampicillin- 100µg/ml and tetracycline- 6µg/ml) followed by 2 hours of incubation at 37°C in shaking condition. After 2 hours of incubation, dsRNA production was induced by adding 10µM Isopropyl β- d-1-thiogalactopyranoside (IPTG) followed by another 3-4 hours incubation stage at same condition as previously done. The samples were stored in -80°C.

3.10 RNA Extraction from Bacterial Culture

The bacterial cultures were centrifuged for 10 minutes and the supernatant was discarded. The pellet was resuspended with Trizol (3 times volume of the pellet) and RNA extraction was executed using Direct-zol™ RNA Kits from Zymo Research. The samples were eluted with 100µl nuclease- free water, the concentration was estimated by Nanodrop and was stored at -80°C.

3.11 Fruit Assay (FA)/Detached Leaf Assay (DLA)/Spike Assay (SA)

Five fresh cherry tomatoes per treatment were collected to experimenting the silencing of target genes of *Botrytis*. For silencing the four target genes of *Fusarium* five wheat leaves of 3-week old and spikes at anthesis period were collected. The fruit/leaf/spike samples were kept on mesh in a box containing wet paper for creating humid environment (relative humidity approximately 85%) inside the box to facilitate the infection in 20°C in light conditions.

Each fruit of fruit (Tomato)/leaf (Wheat)/spike (Wheat) was dipped or sprayed with 100µl containing 5µg of total RNA solution containing dsRNA targeting seven genes (*BcCyp51, BcsdhB, BcTubA* genes for *Botrytis* and *FgCyp51, FgsdhB, FgBt1, Fgbt2* genes for *Fusarium*). After four hours, *Botrytis* inoculation was done with 20 µl containing 3 x 10^6 conidia/ml for Tomato fruit and *Fusarium* inoculation was done with 20 µl containing 20 x 10^4 spores/ml Wheat leaf and spike. Infection was recorded seven days post- inoculation (dpi) for both *B. cinerea* and *F. graminearum*.

3.12 Plant Material

Fresh cherry tomato was bought every week to experiment. The spring wheat seeds were primarily soaked in water on petridish and the germinated seeds were collected for sowing in soil. Single seeds were sown in each pot (8 x 8 x 8) containing sterile soil. The wheat plants were grown in a greenhouse in 20°C temperature, 68.7% relative humidity and 162µmol m^{-2} s^{-1} photosynthetic photon flux density. Every week each block of plants was fertilized with both macro and micro nutrients.

3.13 Fungal Strains and Culture

Fusarium graminearum strain PH1 were maintained in V8 media containing Ampicillin and for sporulation carboxymethyl cellulose (CMC) media (7.5g of CM cellulose yeast extract broth, 0.5g of yeast extract, 0.25g of MgSO4. 7H20, 0.5g of NH4NO3, 0.5g of KH2PO4 dissolved in 1L of distilled water) was used. Every week, a few plugs of growing *Fusarium* mycelium was taken into 50ml of CMC media in 150ml of conical flasks and kept at 28°C at 200 rpm with continuous illumination for 7 days. *Botrytis cinerea* B05.10 was maintained in solid V8 media (10% vegetable juice, 1.05% Bacto- agar, 0.15% $CaCO_3$ and 100ug/ml Ampicillin) at 20°C under constant illumination.

4. RESULTS

4.1 Plasmid Extraction and Gel Purification

Plasmid L4440 containing bacterial culture (three replicates) was cultured overnight at 37°C and the following day plasmid was extracted from the bacterial culture. The concentration was found 64.133ng/µl, 127ng/µl, 107 ng/µl measured by Nanodrop. The samples were stored in -20°C. The extracted plasmid was purified and the concentration was found 52ng/µl, 65.238 ng/µl and 123.113 ng/µl.

4.2 DNA Extraction of *B. cinerea* and *F. graminearum*

B. cinerea and *F. graminearum* pathogens were cultured in Luria Broth (LB) liquid medium. DNA was extracted from both pathogens and the concentration was measured as 15.5ng/µl, 9.39ng/µl for *Botrytis* and *Fusarium* respectively.

4.3 PCR Reaction and Purification

After DNA extraction from *Botrytis* and *Fusarium*, PCR was carried out using their respective primers (Supplementary Table 1). PCR was confirmed by the presence of bands in agarose gel. Then, PCR products were purified and their concentration was recorded by Nanodrop (Table 4.1).

Table 4.1. Concentration of PCR products of DNA extracted from *Botrytis* and *Fusarium*

No	Sample	Concentration (ng/µl)	260/280	260/230
1	*BcCyp51*	47.220	1.74	1.14
2	*BcsdhB*	29.080	2.10	2.33
3	*BcTubA*	25.073	1.72	2.36
4	*FgCyp51*	22.968	2.37	2.08
5	*FgsdhB*	26.502	1.89	2.13
6	*FgBt1*	12.785	2.37	2.50
7	*FgBt2*	36.787	1.98	2.17

4.4 Restriction Digestion, Ligation and Transformation

The PCR products and plasmids were digested with restriction enzyme (Table 3.2). After restriction digestion ligation was carried out in ratio of 3:1 of vector: insert, where 50ng of vector was used in ligation. Following ligation step, transformation of the recombinant plasmid carrying our desired insert targeting respective dsRNA into HT115(DE3) was executed and were plated in LB agar plates for overnight culture in 37°C. The transformation was confirmed by sequencing.

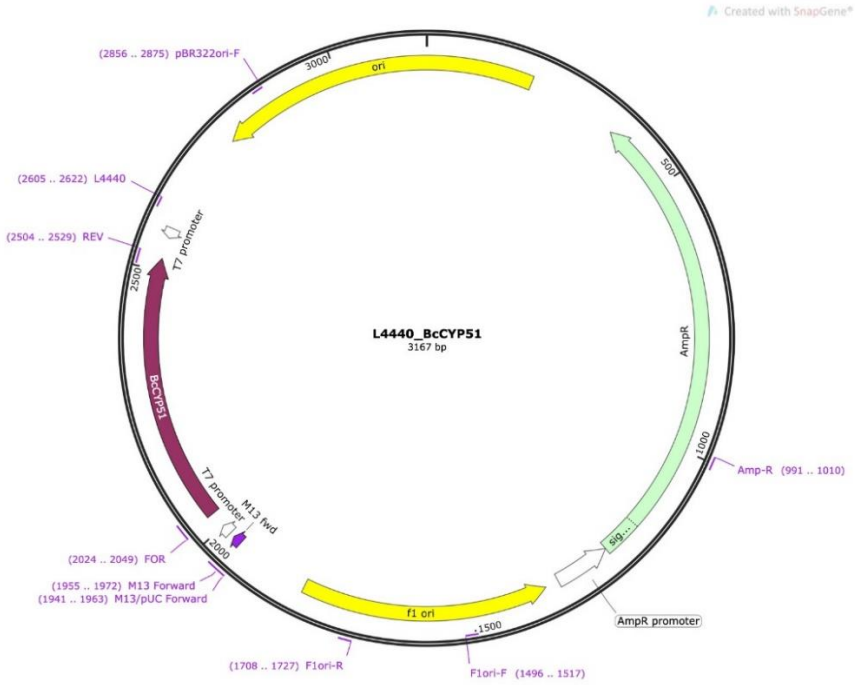

Figure 4.1. Cloning map of recombinant plasmid L4440 containing *BcCyp51* complementary sequence

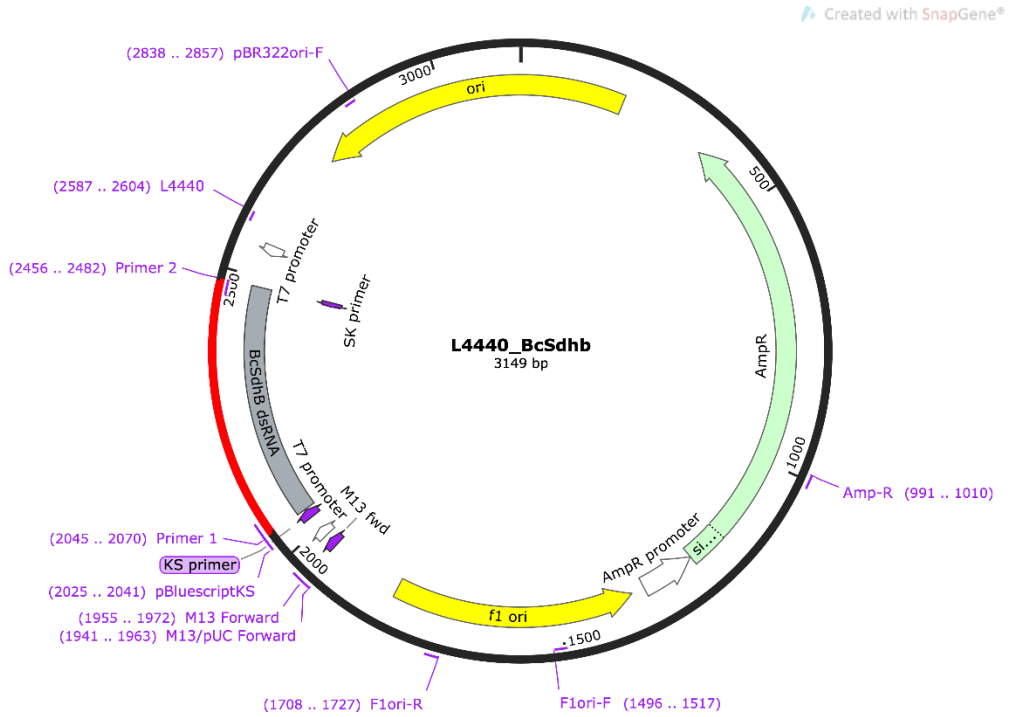

Figure 4.2. Cloning map of recombinant plasmid L4440 containing the complementary sequence of *BcsdhB*

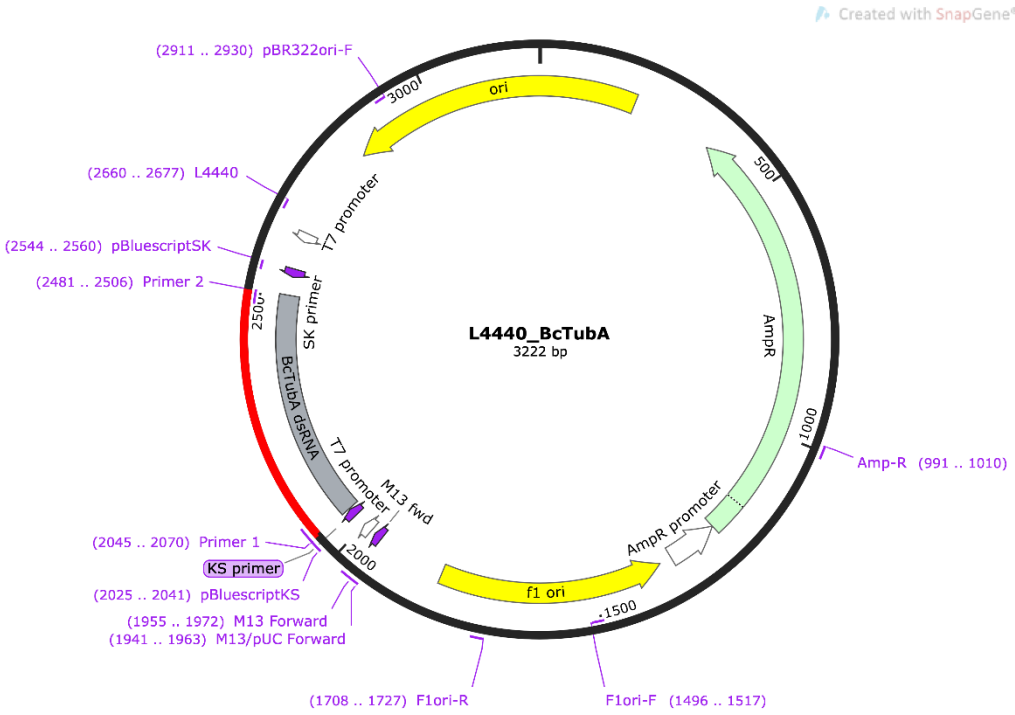

Figure 4.3. Cloning map of recombinant plasmid L4440 containing the complementary sequence of *BcTubA*

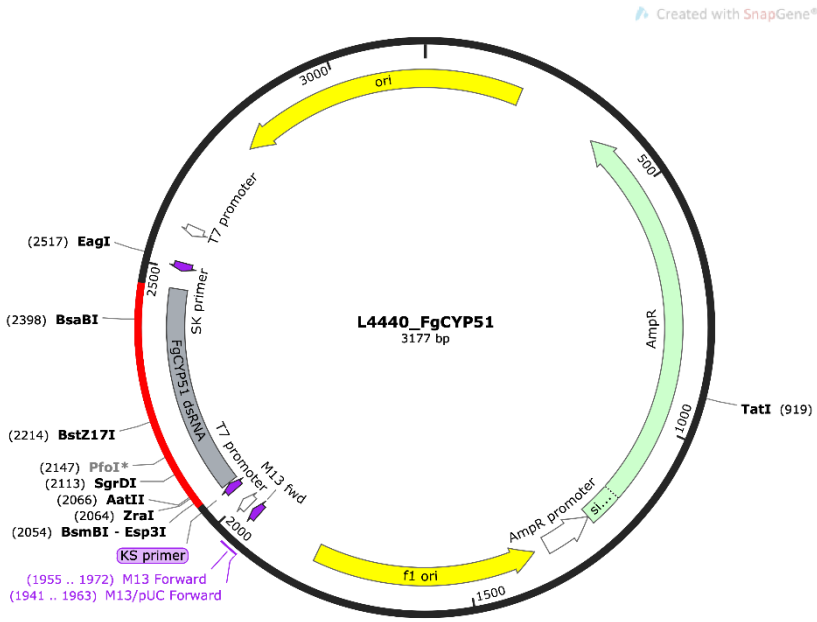

Figure 4.4. Cloning map of recombinant plasmid L4440 containing the complementary sequence of *FgCyp51*

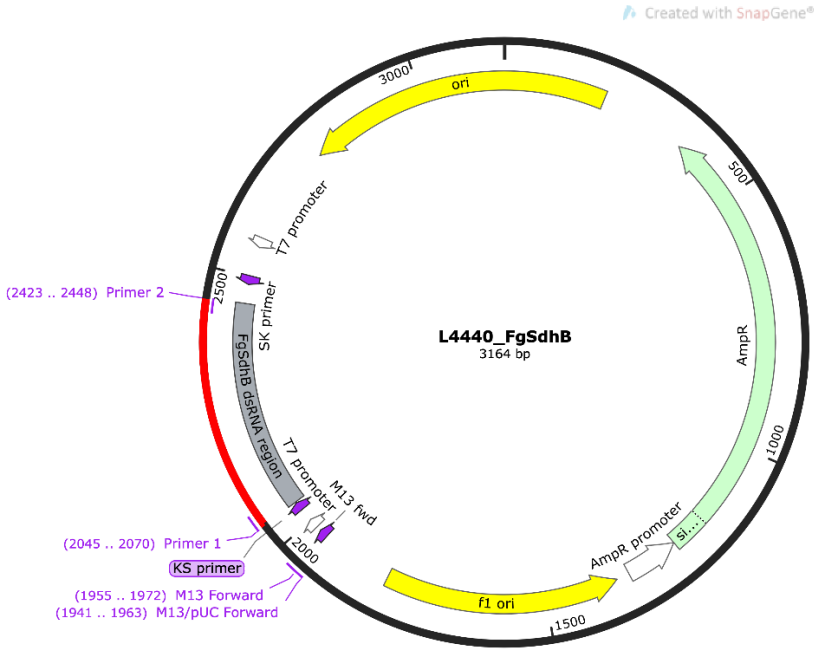

Figure 4.5. Cloning map of recombinant plasmid L4440 containing the complementary sequence of *FgsdhB*

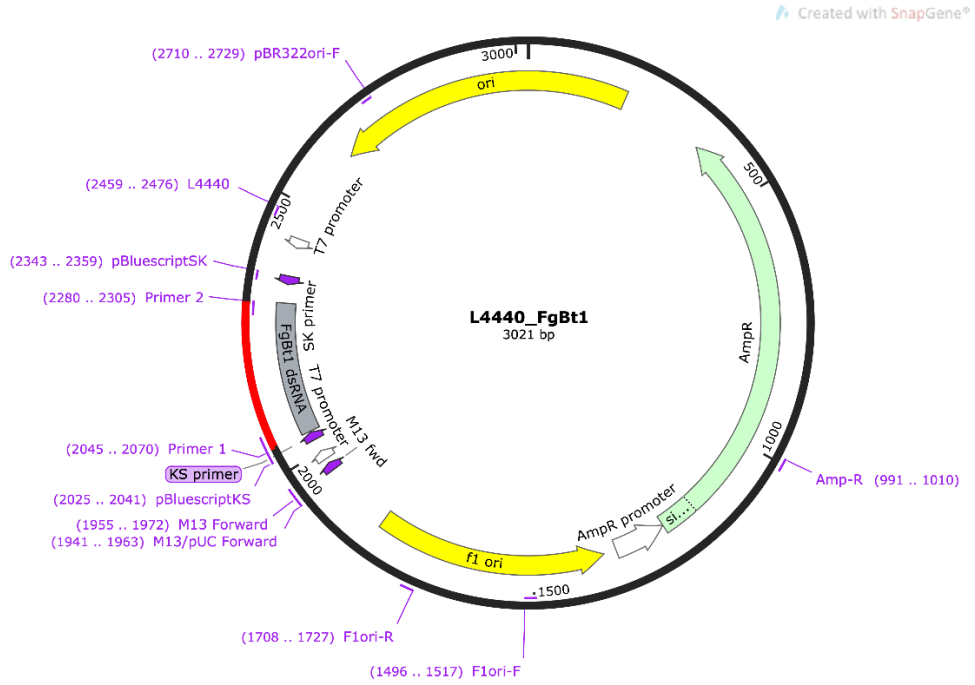

Figure 4.6. Cloning map of recombinant plasmid L4440 containing the complementary sequence of *FgBt1*

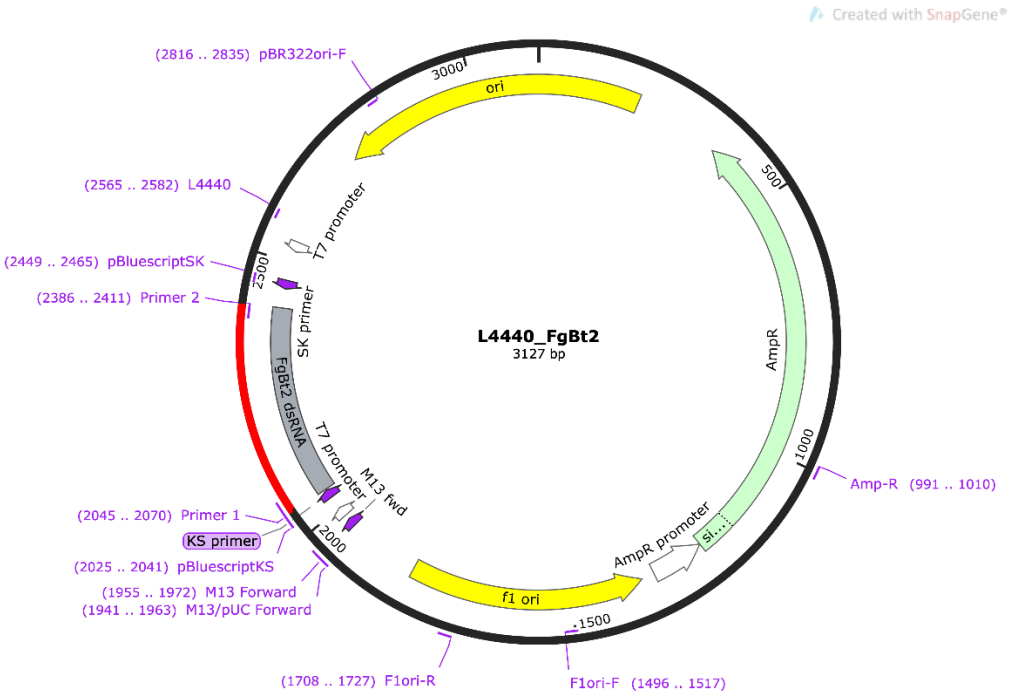

Figure 4.7. Cloning map of recombinant plasmid L4440 containing the complementary sequence of *FgBt2*

4.5 IPTG Induced dsRNA Production by Bacterial Culture and RNA Extraction

Bacterial cultures containing recombinant plasmid of each sample were collected in liquid LB broth containing ampicillin and tetracycline and let the bacteria grow in liquid medium overnight at 37°C in shaking condition. By overnight culture of bacteria, OD_{600nm} was measured and the OD_{600nm} was adjusted in 0.2 by liquid LB broth and kept for 2 hours of incubation at 37°C. Later, IPTG was added to induce dsRNA production by activating the T7 promoter for RNA transcription. RNA from each samples of bacterial culture was extracted. The presence of dsRNA was confirmed by 1% agarose gel (1g of Bacto Agar dissolved in 100ml of 1x TAE buffer) (Figure 4.8).

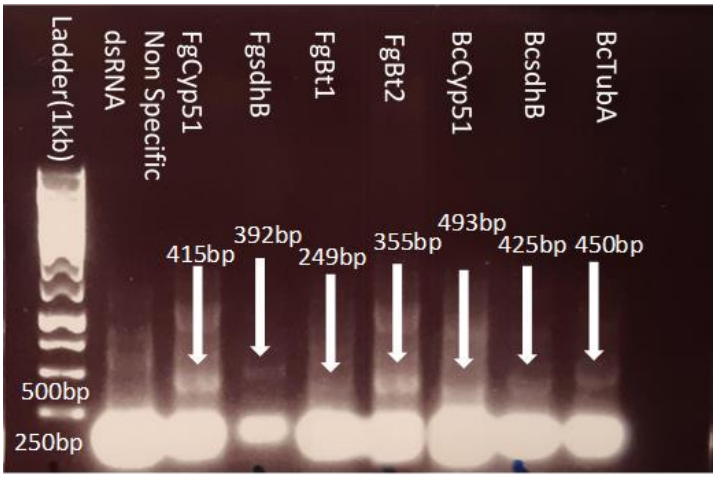

Figure 4.8. **Confirmation of dsRNA band extracted from bacterial culture in gel electrophoresis**. *E. coli* was transformed with a recombinant plasmid containing insert of desired dsRNA sequence. IPTG induced dsRNA was produced in bacterial culture and RNA was extracted from the culture. The presence of dsRNA was confirmed by bands observed in 1% agarose gel. Gene ladder (1kb) was used to confirm the band sizes of dsRNAs and gel red (fluorescent nucleic acid dye) was used to visualize the bands. Arrows indicate the desired bands along with their respective band size.

4.6 Fruit Assay (FA) by Extracted RNA From Bacterial Culture

Three tomato fruit replicates were used for each treatment with two controls (Water and Nonspecific dsRNA). Each tomato fruit was dipped with 5µg of RNA solution containing dsRNA. Four hours post- application of RNA solution, *B. cinerea* conidia inoculation was performed with 20µl containing of $3x10^6$/ml conidia. Infection was recorded on seventh day post- inoculation (dpi). All fruits were kept on mesh having wet cloth inside the box for the purpose of producing humid condition inside box. dsRNA targeting *BcCyp51* and *BcsdhB* genes exhibited reduced infection in comparison to control (Figure 4.9). Although dsRNA targeting *BcTubA* didn't exhibit similar reduced infection, it shown that the dsRNA shows less infection in comparison to control. Combination of all three dsRNAs targeting the three genes (*BcCyp51, BcsdhB, BcTubA*) also exhibited reduced level of disease than control (Figure: 4.9).

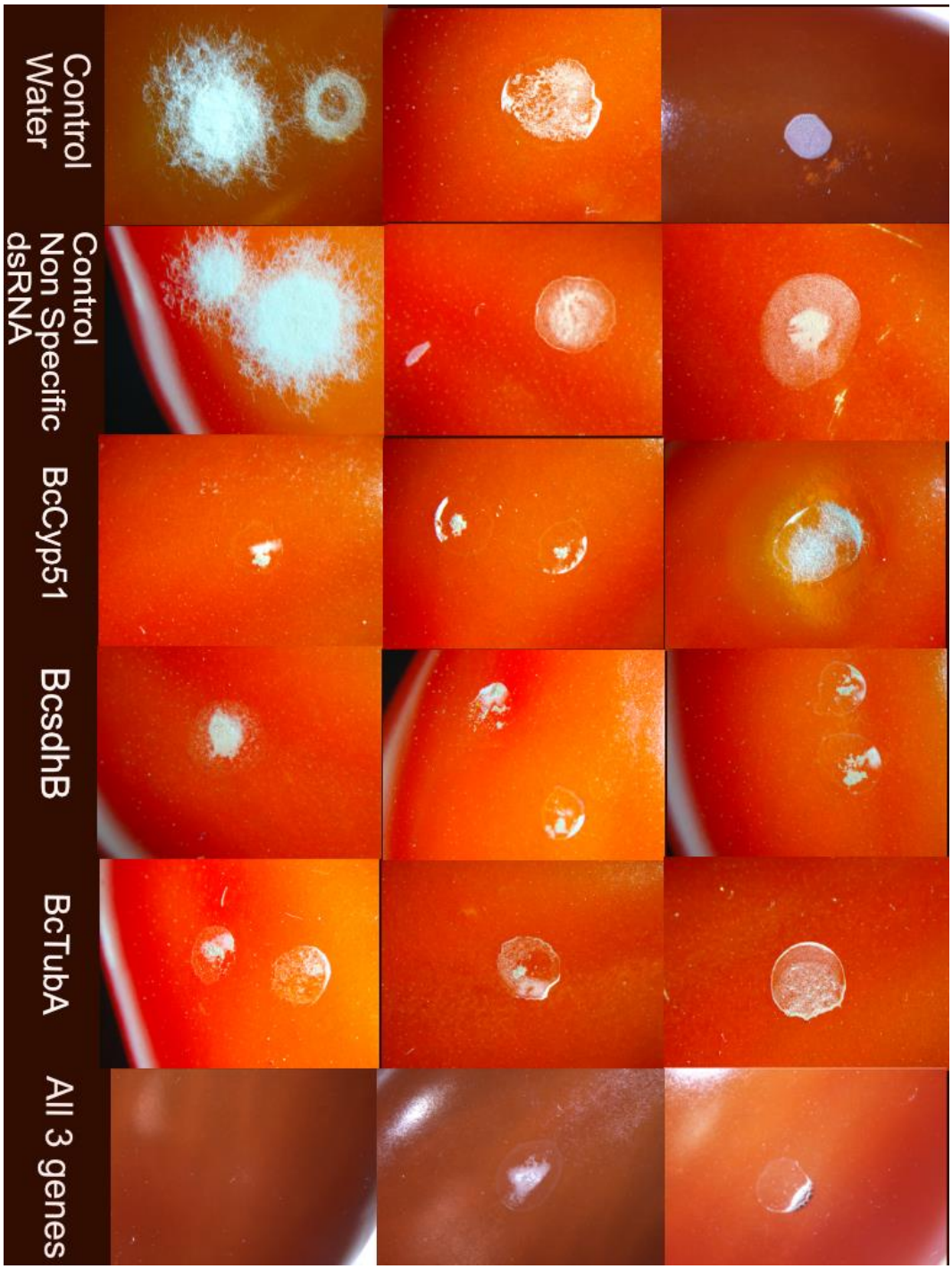

Figure 4.9. Fruit assay for *B. cinerea* infection on tomato. Water and Non-specific dsRNA dipped tomato fruits were used as control. Each tomato fruit was dipped in 5µg of RNA solution containing dsRNA targeting *BcCyp51*, *BcsdhB*, *BcTubA*. Four hours of dsRNA application was followed by fungal inoculation. Infection was recorded seven days of post-inoculation (dpi). Images were taken by stereomicroscope at 1x magnification.

4.7 Detached Leaf Assay and Spike Assay by Extracted RNA From Bacterial Culture

Five young detached leaves/spike were collected for each treatment including two controls (Water and Non-specific dsRNA) and each leaf/spike was sprayed with 100µl of 5 µg of total RNA solution with high pressure containing dsRNA. After four hours of spraying of RNA solution, each leaf/spike was inoculated with 20µl of spore suspension of *F. graminearum* containing $2x10^4$ spores/ml. All the leaves/spikes were kept on mesh in a box containing wet cloth to maintain humidity inside a box.

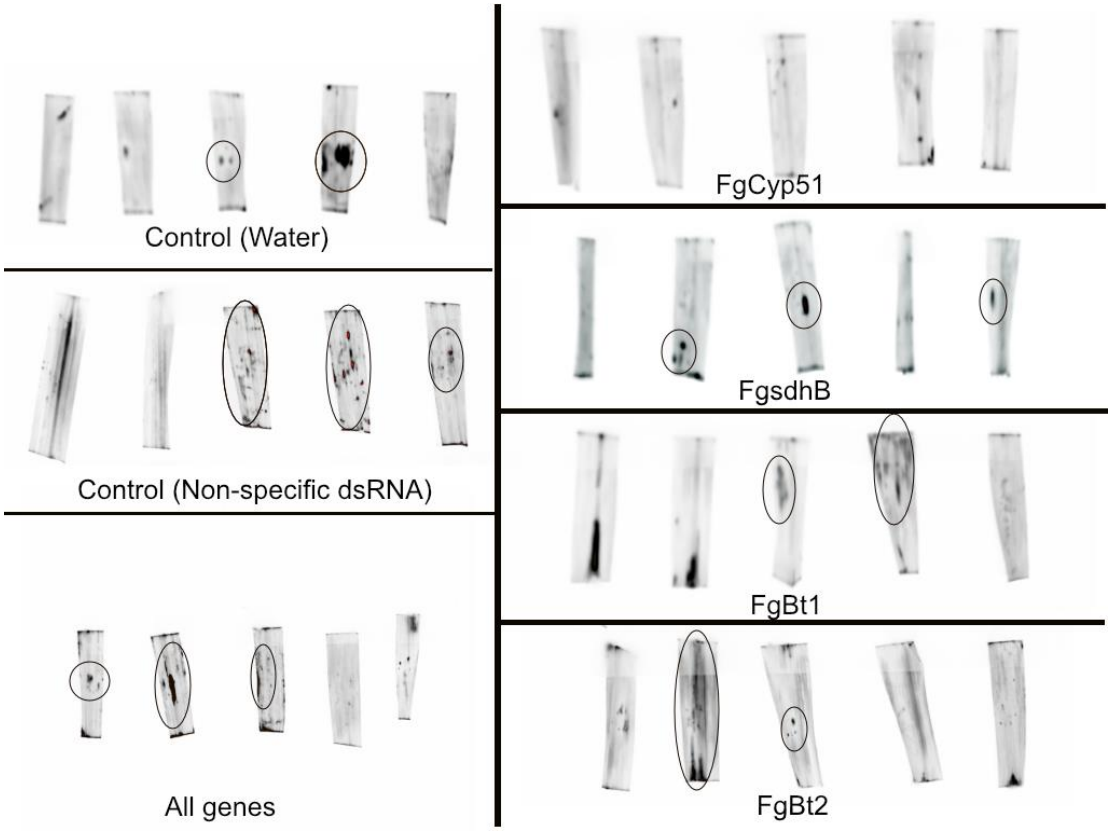

Figure 4.10. Detached leaf assay for *F. graminearum* on wheat detached leaves. Water and Non-specific dsRNA sprayed detached wheat leaves were used as control. Each leaf was sprayed with 5µg of total RNA solution containing dsRNA targeting *FgCyp51*, Fg*sdhB, FgBt1* and *FgBt2*. Four hours of dsRNA application was followed by fungal inoculation. Infection was recorded seven days of post-inoculation (dpi). Images were taken by Bio-Rad ChemiDoc at respective wavelengths for Cy7.

Infection was recorded at seven dpi by Biorad (Zahid et al., 2021). Compared to control (water and non-specific dsRNA) dsRNA targeting *FgCyp51* exhibited significantly less or no infection. dsRNA targeting *Fgbt2* exhibited less or no infection in four leaves. In contrast, dsRNA targeting *FgsdhB, FgBt1* and combination of all genes showed no significant difference in infection level compared to control (Figure 4.10).

In case of Spike assay, infection was observed in all treatments but dsRNA targeting *FgBt1*, *FgBt2* and a combination of dsRNAs targeting all genes exhibited less disease than control (Non-specific dsRNA) (Figure 4.11).

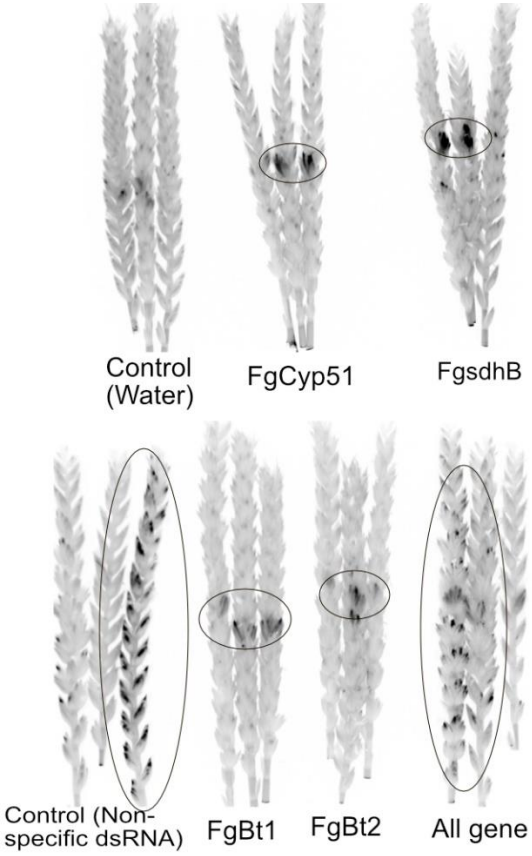

Figure 4.11. Detached spike assay for *F. graminearum* on wheat detached spikes. Water and Non-specific dsRNA sprayed detached wheat spikes were used as control. Each spike was sprayed with 5µg of RNA solution containing dsRNA targeting *FgCyp51*, Fg*sdhB*, *FgBt1* and *FgBt2*. Four hours of dsRNA application were followed by fungal inoculation. Infection was recorded seven days of post-inoculation (dpi). Images were taken by Bio-Rad ChemiDoc at respective wavelengths for Cy7.

5. DISCUSSION

RNAi mediated SIGS technology: basically, a sequence-specific methodology, can be an alternative method to chemical or transgenic disease control technology in agriculture by suppressing or silencing the target gene expression of the pathogen (Dutta et al., 2014; Al-Ayedh et al., 2016). To provide evidence for the potentiality of the SIGS mechanism in the aspect of disease control, the present study was conducted on *B. cinerea* on Tomato and *F. graminearum* on wheat.

Gradual development in research for the production of dsRNA along with low cost has been a focus to scientists to make it easily accessible to farmers (Ahn et al., 2019). Presently, producing dsRNA with a bacterial system has paved the way for an easy and economical method to produce dsRNA compared to dsRNA production by expensive kit (Lisa et al., 2001; Terpe, 2006; Chen et al., 2019b; Niño-Sánchez et al., 2021). A study was conducted to produce dsRNA in both in vivo and in vitro, proving that the cost of in vivo dsRNA production was reduced to one third compared to *in vitro* dsRNA production (Ongvarrasopone et al., 2007). In the current project, dsRNA targeting different genes was produced by bacterial system ranging from 5µg/µl to 12.54µg/µl from bacterial culture.

In the current study, infection of *B. cinerea* was observed in RNA solution containing dsRNA treated tomato fruits. Infection of dsRNA treated fruits was compared with two controls (water and non-specific). The infection area in dsRNA (*BcCyp51, BcsdhB, BcTubA*) treated fruits was restricted compared to controls. Infection was significantly less or absent when the dsRNA targeting all three genes (*BcCyp51, BcsdhB, BcTubA*) was applied together to tomato fruit. Similar attenuated fungal growth was exhibited by silencing *Bc-DCL1* and *Bc-DCL2* genes in Strawberry, Tomato and Arabidopsis exploiting SIGS (Wang *et al*., 2016). Another study (McLoughlin et al., 2018) has also experimented with infection level of *B. cinerea* in dsRNA treated detached leaf of *Brassica napus* and found reduced fungal lesion in leaves affirming the efficiency of SIGS on *B. cinerea*.

In case of wheat, the infection level in *FgCyp51* dsRNA treated detached leaves appeared to be less infected than controls. In case of wheat spikes treated with dsRNA targeting *FgBt1*, *FgBt2* and combination of all four dsRNAs targeting *FgCyp51*, *FgsdhB*, *FgBt1* and *FgBt2* showed minor infection in comparison to non-specific dsRNA control. But further research is required to perceive a precise scenario in case of dsRNA targets and their effectiveness on wheat leaves and spikes. Previously, Koch *et al*. (2016) proved SIGS efficiency by targeting genes (*CYP51A*, *CYP51B*, and *CYP51C*) of *F. graminearum* on Barley detached leaves. The dsRNA targeting the *CYP51* genes disrupted the integrity of the fungal membrane of F. graminearum, thus reducing the infection in leaves that was later confirmed by quantitative PCR.

6. CONCLUSION AND FUTURE RECOMMENDATION

Spray induced gene silencing is an emerging novel method to control plant diseases and a potentially contribute to sustainable development goals (SDG). SIGS technology avoids the complexity of transgenic plants and expensive production. Controlling plant diseases by employing SIGS has widened up the new era of the plant protection arena. Nevertheless, there has been underlying mechanisms, functions in RNAi that requires further investigation. Delivery of dsRNA into plants still requires more research since dsRNA is subject to degradation by RNase activity. In parallel, protocols have to be established for SIGS for applying for different pathogens and host plants by deep study on pathogen morphology and plant-pathogen interaction. Although numerous researches have been conducted on RNAi based SIGS and HIGS, yet utilizing the technology at the field- level still needs to be optimized. Furthermore, several factors at field level can affect the efficacy of dsRNA mediated disease control method. Thus, the aspects are entailed to be considered in future research RNAi arena.

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Reemana FATEMA

CURRICULUM VITAE

Reemana Fatema, from Bangladesh, did her Bachelor in Agriculture (2014) and 1st Master degree in Genetics and Plant Breeding (2016) from Sher-e-Bangla Agricultural University in Bangladesh. Later, she had also accomplished Erasmus Mundus Joint Master Degree in Plant Breeding (emPLANT) from Swedish University of Agricultural Sciences (Sweden) and Ege University (Turkey) from 2019 to 2021. Earlier in 2017, she had worked as English Language Trainer in Bangladesh.

Poster Presentation:

1. Fatema R, Kalyandurg P.B, Vetukuri R.R. (2021). Spray Induced Gene Silencing. Presented in 2021 edition of Thesis Day's poster exhibition at SLU. Held in May 27, 2021 at Swedish University of Agricultural Sciences Link: https://student.slu.se/en/studies/degree-projects/thesisday/2021-posterexhibit/spray-induced-gene-silencing/

2. Fatema R, Islam M.T, Bhuyian M.R.S Rahman J. (2018). Evaluation of Kidney Bean (Phaseolus vulgaris L.) Genotypes Available in Bangladesh for Nutritional Traits and Yield. Poster presented in: 10th Asian Federation of Biotechnology Regional Symposium. Held in Jan 27- 29, 2018 at Dhaka University, Dhaka.

APPENDIX

Supplementary Table 1: Gene name, accession number, primers and dsRNA size

No.	Gene Name	Accession Number	Primers	dsRNA size
1	*Botrytis cinerea* B05.10 Cyp51 (*BcCyp51*)	XM_001549911.2	Bc_Cyp51_XhoI.FOR Bc_Cyp 51_SacI.REV	493
2	*Botrytis cinerea* succinate dehydrogenase iron sulphur protein gene (*BcsdhB*)	XM_001549911.2	Bc sdhB_HindIII_FOR Bc sdhB_SpeI.REV	425
3	*Botrytis cinerea* B05.10 BctubA (*BctubA*)	XM_024690731.1	Bc B05.10BctubA_HindIII.FOR Bc B05.10BctubA_SpeI.REV	450
4	*Fusarium graminearum* PH-1 cytochrome 450 51 (*FgCyp51*)	XP_011321548.1	Fg Cyp51A_HindIII_FOR Fg Cyp51A_SpeI.REV	415
5	*Fusarium graminearum* PH-1 succinate dehydrogenase iron-sulfur protein (*FgsdhB*)	XP_011324170.1	Fg PH1_HindIII_FOR Fg PH1_SpeI_REV	392
6	*Fusarium graminearum* beta tubulin 1 (*Fgbt1*)	FGSG 09530.3	Fg Bt1_HindIII.FOR Fg Bt1_SpeI.REV	249
7	*Fusarium graminearum* beta tubulin 2(*Fgbt2*)	FGSG 06611.3	Fg Bt2_HindIII.FOR Fg Bt2_SpeI.REV	355

Supplementary Table 2: Primer sequences used for dsRNA synthesis

No.	Primer Name	Sequence	Size
1	Bc_Cyp51_XhoI.FOR	aaggctcgagTGTTTTGACAACCCCCGTAT	493
2	Bc_Cyp 51_SacI.REV	aaagagctcTGTAAGACGAGCGCATCAAC	
3	Bc sdhB_HindIII_FOR	aaaaaagcttCAGAACTCAGATGCGAACCA	425
4	Bc sdhB_SpeI.REV	aaaaactagtCATAGGTGTGGGGTAGTGGG	
5	Bc B05.10BctubA_HindIII.FOR	aaaaaagcttTACCACCTGTCTCCGTTTCC	450
6	Bc B05.10BctubA_SpeI.REV	aaaacccgggGTGAATTGATCACCGACACG	
7	Fg.Cyp51A_HindIII_FOR	aaaaaaagcttCCTCTTTTAGCACGGAGACG	415
8	Fg.Cyp51A_SpeI.REV	aaaaaactagtCTCGGCAGTGAGTTTTCTCC	
9	Fg PH1_HindIII_FOR	aaaaaaagcttGGACCTTGTCCCTGATCTGA	392
10	Fg PH1_SpeI_REV	aaaaacccgggGCTTCTTGATCTCGGCAATC	
11	Fg Bt1_HindIII.FOR	aaaaaagcttGGTCAGTGCGGTAACCAAAT	249
12	Fg Bt1_SpeI.REV	aaaacccgggGAAAACGAAGTTGTCGGGTC	
13	Fg Bt2_HindIII.FOR	aaaaaagcttTGTGACGACATGTTTCCGAT	355
14	Fg Bt2_SpeI.REV	aaaacccgggGACGGAAGTTTGGACGTTGT	