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External RNA interference against the fungus *Cercospora zeina*

by

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Declaration of originality

I _____________Ingrid Marais_______________declare that the thesis, which I hereby submit for the degree ______MSc. Biotechnology_________________ at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

Date: ______10/12/2020_______________________

Ethics statement

The author, whose name appears on the title page of this dissertation, has obtained, for the research described in this work, the applicable research ethics approval number **NAS230/2019**.

The author declares that he/she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

Synopsis

Globally crop production is under threat due to devastating fungal pathogens and the incorrect management thereof. RNA interference (RNAi) is a novel way in which fungal diseases can be controlled in an environmentally friendly way. This study focuses on the maize yield limiting fungus, *Cercospora zeina*, observing if the fungus is able to take up externally applied double stranded RNA (dsRNA), and if this RNA is able to have an impact on gene expression and development. RNAi genes within Dothideomycete fungi were identified and the phylogenetic relationship of these genes were analysed and compared. *Cercospora zeina* contained orthologs to all the major RNAi machinery proteins. Different RNAi strategies against fungi is discussed within this study. *Cercospora zeina* was shown to take up externally applied dsRNA. Two dsRNA constructs were created which targeted either the GFP gene within a transgenic *C. zeina* fungus, or growth and pathogenicity genes within a wild type *C. zeina.* The GFP fluorescence was observed after the fungus was treated with the GFP targeting dsRNA construct and a decrease in fluorescence of up to 50% was observed. The gene expression of GFP and the three other target genes were also analysed and a decrease of 50% was observed within the GFP expression as well as the chitin synthase *C. zeina* gene expression. The metabolic activity of *C. zeina* was also significantly decreased, by 40%, after the addition of dsRNA. Future prospects of using this type of RNAi bio-fungicide will be discussed within this study and considerations for such a novel technology will be addressed. This study forms the basis for the development of a potential RNAi fungicide against the yield limiting pathogen, *Cercospora zeina*.

Preface

The research conducted in this thesis was done in the Molecular Plant-Pathogen Interactions (MPPI) research laboratory in the Plant Sciences Complex at the University of Pretoria, South Africa.

The overall global population is constantly increasing by a rapid rate, where it is projected that the world population will reach 9.7 billion in the year 2050 and 11.2 billion in the year 2100 (FAO, 2017). Meeting this growing demand, the agricultural sector in 2050 will need to produce 50 percent more food, feed and biofuels than produced in the year 2012 (FAO 2017). This increase in demand could ultimately lead to natural resources used for agriculture becoming scarce and those available will be overexploited and used in an unsustainable manner leading to environmental degradation (FAO 2017). It is thus of great importance that sustainable agricultural practices will be developed and used to create a stable, resource rich future for the world population.

Cereal grains constitute more than 50% of the human daily calorie intake, with the major staple grains being wheat, rice and maize and to a lesser extent other grains such as sorghum and millets (Awika, 2011). Cereals are one of the most important food commodities, not just for human consumption, but largely for livestock feed, with the demand for feed increasing as the global diet shifts to more meat and dairy-based products (Alexandratos and Bruinsma, 2012). In 2016, the FAO showed that since the 1990s, on a global level, there was only a slight increase of more than 1% in grain yield, much lower when compared to the 1960s. Due to the increasing food demand in the coming decades, crop yield increases on the existing land areas are substantially important.

Direct crop yield losses due to pathogens, pests and diseases on a global scale has been shown to be between 20 to 40% (Oerke et al., 2012; Savary et al., 2012). Plant microbial diseases account for up to 16% of the global crop yield losses, with 70 to 80% of these losses caused by fungal diseases (Moore et al., 2011). Some of the most devastating fungal diseases on cereal crops include rice blast disease caused by *Magnaporthe oryzae,* wheat rust diseases caused by *Puccinia* spp*.,* as well as ear rots caused by *Fusarium graminearum* and *Fusarium verticillioides* (Dean et al., 2012).

Fungal crop diseases are mainly managed using fungicides. Chemical fungicides are classified based on their mobility within the plant (systemic or contact) and their modes of action (McGrath, 2004). Most fungicides only function as preventative fungicides, meaning that they have to be applied before any disease infection, thus limiting their overall effectiveness in disease established crops (Vincelli 2002). The problem with chemical fungicides is resistance development within the pathogen, the fact that they are not all biodegradable, as well as the harmful environmental effects fungicides might have, such as toxicity to amphibian species (Belden et al., 2010; Johansson et al., 2006; Vincelli, 2002). This led researchers to look at alternative approaches to fungal crop protection, including using an external RNA interference (RNAi) approach (Wang et al., 2016; Koch et al., 2016). Spraying dsRNA, which

targets specific fungal genes, onto infected plants could lead to a decrease in infection, and ultimately healthier plants with better yields.

Maize is a very important staple food crop with regards to global nutrition, and especially African nutrition (Nuss and Tanumihardjo, 2010). In the year 2018/2019, 1.09 billion metric tons of maize was produced worldwide, making it the most produced crop in the world (Shahbandeh, 2019). Maize crop is used largely for animal feed, also human consumption and for the production of biofuels (Nuss and Tanumihardjo, 2010; Ranum et al., 2014). The global population is rapidly increasing the demand for staple food crops such as maize. Maize production is unfortunately threatened by a variety of external factors including abiotic and biotic stresses. One of the biggest biotic factors threatening maize yields are fungal diseases, including diseases such as northern corn leaf blight, gibberella ear rot and the one I will be focusing on in this study, grey leaf spot disease.

Cercospora zeina is one of the major maize yield limiting fungal pathogens, occurring in maize producing countries such as the USA, Brazil, Kenya and South-Africa (Goodwin et al., 2001; Meisel et al., 2009; Wang et al., 1998). This pathogen belongs to the Dothideomycete class, which contains a variety of important plant pathogens including, *Cladosporium fulvum,* the devastating tomato pathogen (Ohm et al., 2012). *Cercospora zeina* is the causal agent of grey leaf spot disease (GLS) in South-Africa (Meisel et al. 2009). GLS causes necrotic lesions on the maize leaf blade leading to major yield losses where in severe cases up to 65% loss was reported (Ward 1996; Meisel et al. 2009). GLS is mainly managed using strobilurin fungicides which reduces the respiration capacity of the fungus, but resistance development to these fungicides are high, increasing the need for novel fungicides which does not cause such high resistance development within its target fungi (Vincelli 2002; Fernández-Ortuño et al. 2006; Wise 2014).

Presently there is no fullproof method for controlling GLS disease in maize, with no maize lines showing total resistance against the fugus or fungicides that will completely destroy this GLS causing fungus. This study will discuss the different novel ways in which fungal pathogens can be controlled using RNAi strategies and will look at a strategy specifically aimed against the fungal pathogen *Cercospora zeina*.

RNAi research has become increasingly more important over the years, with Web of Science Core Collection showing between 2000-2020 that there have been 1058 publications on RNAi within plants. The number of publications published about RNAi within fungi was 207, where 18 of these had to do with externally applied RNAi. The majority of these publications were published within the period 2016-present, thus showing that this is a new emerging important field of research.

The **overall aim** of this study was to evaluate the impact that dsRNA has on *Cercospora zeina* gene expression *in vitro.* The null hypothesis of the study was that the dsRNA would have no significant effect on *C. zeina* specific gene expression.

Chapter one is a literature review that will focus on the pathogen *Cercospora zeina,* which causes grey leaf spot disease and how it is managed, looking especially at fungicide usage. This chapter was written following guidelines given by Fungal Biology Reviews. The chapter will discuss how the RNAi process works in organisms, also focusing specifically on fungi. The fungal RNAi machinery within different Dothideomycetes, including *Cercospora zeina*, is also analysed using a phylogenetic approach. In this chapter a brief overview of what has been done in other external RNAi studies will be given and lastly the potential of using such products will be discussed.

Chapter two will cover the experimental research on RNAi against *C. zeina* and the results that have been obtained. This chapter is written based on the guidelines given by the journal, Fungal Biology. Specific *C. zeina* genes important within the growth and pathogenicity of the fungus were chosen as targets for the RNAi process. The optimal conditions for silencing a GFP gene in a transgenic *C. zeina* strain is shown. In this chapter the expression results of *C. zeina* genes after treatment with dsRNA is also shown. Lastly, the impact that the dsRNA treatment has on *C. zeina* metabolic activity is assessed.

Chapter three is the concluding chapter which deals with future prospects of using RNAi commercially. The different criteria which needs to be taken into account when deciding to do an RNAi experiment is discussed. Possible future RNAi target genes within fungi are identified. Advancements and new technologies such as Unmanned Aerial Vehicle (UAV) and nanotechnologies used together with RNAi is discussed. Commercially available RNAi products is also mentioned within this chapter.

The following outputs have been generated from this study

- **2019 International Society for Molecular Plant-Microbe Interactions (IS-MPMI) XVIII Congress, in Glasgow, Scotland**
  (https://apsjournals.apsnet.org/doi/10.1094/MPMI-32-10-S1.1)
    - Abstract submission
    - Poster presentation

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Most importantly I would like to give all credit to my Heavenly Father God. He has answered countless prayers and kept me going when I felt like giving up. (Phil 4:13)

List of Abbreviations

°C	Degrees Celsius
µg/µl	Microgram per microliter
µg/ml	Microgram per milliliter
µl	Microliter
µM	Micromolar
Ago	Argonaute
BLAST	Basic local alignment search tool
cDNA	Complementary DNA
ChsD	Chitin synthase D
CMA	Cornmeal agar
DCL	Dicer-like
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleotide
Ecp2	Extracellular protein 2
ex-siRNA	Exonic small interference RNA
gDNA	Genomic DNA
GLS	Grey leaf spot
HIGS	Host induced gene silencing
Mnase	Micrococcal nuclease
milRNA	Micro RNA-like
miRNA	Micro RNA
mRNA	Messenger RNA
MSUD	Meiotic silencing by unpaired DNA
ng/µl	Nanogram per microlitre
nm	Nanometer
PCR	Polymerase chain reaction
Psd3	Phosphatidyl serine decarboxylase pro-enzyme 3
PTGS	Post-transcriptional gene silencing
QTL	Quantitative trait loci
RdRP	RNA-dependent RNA polymerase
RFU	Relative fluorescent units
RISC	RNA-induced silencing complex
RFU	Relative fluorescent units
RNA	Ribonucleic acid
RNAi	RNA interference
RT-qPCR	Real-time quantitative PCR
SEM	Scanning electron microscope
siRNA	Small interfering RNA
ssRNA	Single-stranded ribonucleic acid
WT-*C. zeina*	Wild-type *C. zeina*
YEPD	Yeast extract potato dextrose

Chapter 1

Literature review:
The potential of external RNA interference to develop novel fungicides against the maize foliar disease Grey Leaf Spot

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This chapter has been prepared in the format of a manuscript for a review journal (Fungal Biology Reviews). I reviewed different literature sources and put together the manuscript. I also identified different RNAi genes from literature and downloaded and organised more than a 100 of these genomes. Dr. T.A. Dong automated the annotations and found the protein ortholog family groups and generated the phylogenetic trees. I edited the trees and interpreted the results from the trees. Prof. D.K. Berger contributed to the strategy of the phylogenetic analysis and both him, Prof. J. Theron and Dr. T.A. Dong critically reviewed and edited this manuscript.

Abstract

Cereal crops are constantly under threat from a wide range of pests and diseases. One of these diseases is the maize crop disease called grey leaf spot which is caused by the fungus *Cercospora zeina*. This disease is managed by using foliar fungicides, which has the disadvantage of causing resistance development. This opened the possibility of developing a novel type of fungicide which utilises the RNA interference (RNAi) mechanism. RNAi is a gene silencing mechanism in which the expression of protein coding genes is regulated. Components of the RNAi machinery are able to move between different kingdoms such as plants and fungi and is called Cross-Kingdom RNAi. RNA interference can be used for various purposes including: (i) studying gene function; (ii) creating transgenic plants which utilize the host induced gene silencing strategy (HIGS) which protects the host against harmful pests and diseases; (iii) degrading certain harmful genes within therapeutics; (iv) using RNAi as a type of pesticide/fungicide to protect plant hosts from damage/disease. In this review the focus will be on RNAi especially within fungi and analysing the possibilities of using this mechanism for the production of a bio-fungicide/pesticides. Different components of the RNAi machinery were identified within *Neurospora crassa*. Orthologs within more than a 100 Dothideomycete fungi were identified and phylogenetic trees were created based on the grouping of the orthologs. *Cercospora zeina* contained orthologs to all of the RNAi machinery proteins, indicating that *C. zeina* can potentially be used within external RNAi studies. The fungus *Zymoseptoria tritici* contained orthologs to all but one of the RNAi proteins, indicating that the RNAi phenomenon developed differently within fungi from the same order. The different RNAi components are discussed and how they affect fungal growth and development will also be covered.

Keywords: RNA interference, *Cercospora zeina*, fungi, fungicide, pathogen

1.1 Introduction

Pests and diseases play a major role in crop yield losses (Oerke, 2006; Savary et al., 2012). One of the most devastating maize yield pathogens is the fungus *Cercospora zeina* which causes grey leaf spot disease. The losses are managed using a variety of strategies including the use of fungicides. Fungicides are not always ideal as they could be harmful to the environment and the fungi is able to develop high degrees of resistance if sprayed continually. This opens a gap for new fungal management practices which are environmentally friendly as well as effective over the long term.

RNA interference (RNAi) is a gene silencing mechanism which targets protein coding genes and which is present in most organisms, including fungi. RNA interference uses a variety of proteins which facilitates the recognition and processing of foreign/endogenous double-stranded RNA molecules which are identical to specific protein coding genes and simultaneously targets these genes for degradation (Agrawal et al., 2003; Billmyre et al., 2013). RNAi can be used for a wide range of

applications, including targeting genes within fungi for degradation through transgenic plants expressing hairpin RNA and even targeting fungal genes for degradation using exogenously applied double-stranded RNA. Utilising this method for pest and disease control has great potential as it is environmentally friendly, and it is gene specific. In this review the different aspects of RNAi will be discussed and how this can be used to create novel pest and disease controls.

1.2. The maize foliar disease Grey Leaf Spot and how it is managed

Grey Leaf Spot disease

Grey leaf spot (GLS) disease is a fungal foliar disease of maize, which occurs in most maize growing countries and was first described in the USA (Tehon and Daniels, 1925; Ward et al., 1999). This disease will cause necrotic, grey lesions on the maize leaf blade which reduces the photosynthetic capacity of the plant, decreasing grain fill and leading to severe yield losses (Figure 1.1). The GLS-causing pathogen was first characterized into two groups, mainly the *Cercospora zeae-maydis* Group I causing disease in the USA, Canada, Mexico and Brazil and *Cercospora zeae-maydis* Group II occurring in the eastern part of the USA and sub-Saharan Africa (Brunelli et al., 2008; Crous et al., 2006; Wang et al., 1998). In 2006, after extensive morphological and phylogenetic analysis of these two groups, the *C. zeae-maydis* Group II was reclassified as *Cercospora zeina* and Group I was classified as *C. zeae-maydis* (Crous et al. 2006).

Figure 1.1: Images of Grey leaf spot disease. Grey leaf spot disease threatens global food security by infecting maize leaf blades, forming necrotic lesions running parallel with the veins, causing a decrease in the photosynthetic capacity of the plant. A, healthy leaf blade without any GLS infection B, Grey tan lesions caused by GLS running parallel to the leaf blade, C, GLS lesions coalescing causing major damage. Photo credit DK Berger

GLS was first reported in South-Africa in 1988 in the KwaZulu-Natal province, and it was later shown that *C. zeina* was the causal agent of disease (Ward et al. 1999; Meisel et al. 2009; Ward 1996). The GLS disease is characterized as one of the most significant yield reducing diseases globally, where in some cases, up to 65 % yield losses have been observed (Ward et al., 1999). Yield losses depend on a

variety of factors including the type of hybrid used, tillage practices and fungicide usage (Ward and Nowell 1998; Ward et al. 1999).

The majority of *Cercospora* species that have been used in population genetics studies have shown that these species contain high population diversity. *Cercospora zeina* in South-Africa was used in a population genetics study where they found an overall population gene diversity of 0.35, which is very high (Muller et al., 2016). A study conducted on the genetic population of *C. zeina* between five different countries in Africa also showed high genetic diversity (Nsibo et al., 2021). It was found that the *C. zeina* Zambian population was distinct from the rest of the populations in Africa, and this could also mean that the pathogenicity of these isolates differs from the other populations (Nsibo et al., 2021). No reports have been published to date about variation in pathogenicity and aggressiveness between different *C. zeina* isolates, so it would be interesting to evaluate if the genetic diversity would influence disease susceptibility or resistance.

The infection strategy of *C. zeae-maydis* was shown by Beckman and Payne in 1982. They infected maize plants with *C. zeae-maydis*, while applying sufficient heat and moisture conditions to the plants. The leaf tissue samples were taken and prepared for scanning electron microscopy (SEM) analysis. From these SEM images it was clear that the fungal appressoria penetrated the maize plant through the stomata and were shown to internally colonize the intercellular spaces within the mesophyll cells (Beckman and Payne, 1982).

The first symptoms of the *C. zeae-maydis* disease was shown to only be visible, 9-12 days after inoculation, forming small chlorotic dots (Beckman and Payne, 1982). The dots will gradually increase in size and will stay parallel within the leaf veins while progressing to maturation (Beckman and Payne, 1982). From the point of inoculation of *C. zeae-maydis* onto the maize plant, up to the point of mature lesions which sporulates, three weeks will pass. Favourable environmental conditions such as warm temperatures between 25 – 30 °C as well as high relative humidity will allow the conidia to germinate and for germ tubes, the germ tube will grow towards the stomata, forming appressoria and then penetrating the plant.

The infection strategy of *C. zeina* has not been studied as extensively as that of *C. zeae-maydis,* but it is believed to occur in a similar way (Meisel et al., 2009). Glasshouse trials of *C. zeina* infection on maize showed that the symptoms also take around 2-3 weeks or more to be visible (Korsman et al., 2012; Meisel et al., 2009; Meyer et al., 2017). Observing the available literature, a link is shown between the physiological development of *C. zeae-maydis* and *C. zeina* on maize and it can be concluded that the infection strategy of both pathogens are also similar.

There is contradiction in the literature regarding the nomenclature of the pathogenic strategy of *C. zeina*. After conducting a Scopus search, five papers mentioned that *C. zeina* is necrotrophic and another five mentioned that it is hemibiotrophic (data not shown). The long latent period exhibited by the fungus

supports using the term "hemibiotroph" to describe *C. zeina*, and this is the preferred term for this MSc dissertation. After the latent period, the *C. zeina* pathogen disrupts its host plant's normal function by causing necrotic lesions parallel to the maize leaf blades, reducing the photosynthetic capacity of the plant and ultimately reducing yields, especially if the lesion coalesces causing blighting to the whole leaf (Meisel et al., 2009; Ward et al., 1999). The pathogen will overwinter on crop debris from previously infected maize plants which remained on the soil surface for the next season. Reduced tillage practices and less frequent crop rotation may increase the amount of infected debris, increasing the prevalence of the disease. GLS has a long latent period of between 14 to 28 days after infection before any lesions can be observed and the conidial spores will germinate when the desired high relative humid conditions persist and will infect the previously healthy maize leaves (Ward et al., 1999).

Management of GLS

GLS is mainly managed using conventional tillage practices, more resistant cultivars, crop rotations as well as fungicide treatment (Ward, 1996). Conservation agriculture as management practice, is not as effective anymore due to the shift in tillage practices, causing a buildup of fungal inoculum. No-till practices have become more popular due to the benefits such as reducing soil degradation, improving nutrient cycling and water conservation (Derpsch et al., 2010; Triplett and Dick, 2008). The problem with the development of resistant cultivars is the quantitative resistant nature of maize against GLS and the development of such hybrids is a very slow process in developing countries (Nowell, 1997; Ward et al., 1999). Fungicide usage needs to be in accordance with specific guidelines, but unfortunately some farmers deviate from the recommended quantity and different spray cycles having a detrimental effect on the environment as well as increasing the risk of resistance development (Brent and Hollomon, 1995; Hobbelen et al., 2014; Sowunmi et al., 2019).

Plants have evolved mainly two types of disease resistance traits including qualitative resistance where individuals are either resistant or susceptible and they segregate as different loci. On the other hand, quantitative resistance is where there is a distribution of phenotypes including resistant to susceptible to varying degrees (Corwin and Kliebenstein, 2017; Poland et al., 2009). GLS is thought to be caused by a hemibiotrophic/necrotrophic pathogen with less specialized infection structures and different virulence factors than a biotroph, rendering single-gene resistance inadequate to protect the host plant (Mendgen and Hahn, 2002). Quantitative resistance plays a large role in the management of GLS and there have been a number of studies which have identified and characterized the mechanisms underlying the quantitative disease resistant loci in the maize genome (Benson et al., 2015; Berger et al., 2014; He et al., 2018). Quantitative resistance can cause a range of specific sub-phenotypes including slowing the rate of infection, reducing the rate at which infected lesions expand, reducing sporulation, increase in the latent period, and an increase in the concentration of the pathogen necessary before establishing successful infection (Benson et al., 2015; Berger, 1977).

Disease resistant quantitative loci (QTL) have been identified for GLS including three QTLs at alleles *qGLS1.04*, *qGLS2.09*, and *qGLS4.05* and also a putative detoxification gene was shown to be upregulated within the mapped QTL regions (Benson et al., 2015). It has been suggested that specific quantitative disease resistance genes are related to biological processes such as flowering time as well as leaf structure (Poland et al., 2011; Wisser et al., 2006). A relationship between the GLS disease development and leaf structure was shown by Benson et al. in 2015. They showed that inter-vein distance (IVD) and disease development was positively correlated due to the fact that lesion development on inbred maize with narrow veins produced fewer inoculum than inbred lines with wider veins. They infected both types of leaf veins with the same initial amount of inoculum and found that the conidiophore counts were higher in the wider veins compared to narrow veins. This type of research helps us to understand what morphological characteristics in plants could play a role in disease resistance, providing new breeding avenues and possibly better management strategies against GLS.

Fungicides

Grey leaf spot disease is managed using resistant hybrids along with fungicides. The success of the fungicide product used depends on the proper timing of the application, the method of application and also the severity of the disease level in the field (Wise, 2014). With GLS management, the most effective timing of fungicide treatment was when the basal leaves were infected before the exponential phase of the disease has occurred (Ward, 1996). However, for the disease to be eradicated further fungicide spray is necessary until the crop matures (Ward, 1996). Fungicides are classified agronomically according to their mode of action. The three main classes of fungicides used against maize foliar diseases in the USA include, the quinone outside inhibitor (QoI)/strobilurins, the DMI Triazoles and the Mix mode of action fungicides, the majority being foliar fungicides (Gupta, 2012; Wise, 2014). Fungicides can either be preventative, applied before disease onset, or curative, applied after disease.

The QoI (strobilurin) fungicides are preventative fungicides which target electron transport at the quinol oxidation site, thus inhibiting mitochondrial respiration (Bartlett et al., 2002; Vincelli, 2002). The discovery of these fungicides were inspired by fungicidal derivatives of β-methoxyacrylic acid found to be produced by certain basidiomycete fungi (Fernández-Ortuño et al., 2008; Kraiczy et al., 1996). Mitochondrial respiration is inhibited through the active ingredient of the fungicide binding at the outer quinol oxidation (Qo) site and preventing the transport of electrons from cytochrome *b* to cytochrome *c* leading to less ATP produced and thus an energy deficiency (Fernández-Ortuño et al., 2008). The cytochrome bc_1 protein is an integral membrane protein and makes up 11 different polypeptides in eukaryotes, operating as structural and functional dimers (Fernández-Ortuño et al., 2006, 2008).

The disadvantage of this fungicide is that it has only one mode of action, thus it is a site-specific fungicide and resistance development by pathogens against the fungicide is a high possibility (Vincelli,

2002). QoI resistance arises from target specific mutations within the cytochrome b gene (*CYTB*) (Fernández-Ortuño et al., 2008). This resistance is easily obtained with a single nucleotide substitution, taking amino acid number 143 from a glycine to an alanine, shown to occur in a variety of phytopathogenic fungi including *Blumeria graminis* and *Mycosphaerella fijiensis* (Fernández-Ortuño et al., 2008; Gisi et al., 2002). The amount of resistance is usually calculated by using a specific concentration as a baseline, which is higher than the minimum inhibitory concentration of the fungicide, and any disease development at such a concentration or higher is classified as resistant. As the risk of resistance development is high, use of the QoI fungicide use should be monitored and limited.

An example of extreme resistance development against this QoI fungicide class was shown in Spain where the cucurbit-powdery mildew, *Podosphaera fusca,* developed high degrees of resistance, in some cases up to 74 %, against QoI fungicides, rendering their use ineffective (Fernández-Ortuño et al., 2006). The *P. fusca* isolates were classified as resistant if they were able to grow after the addition of doses higher than 250 µg/ml of the strobilurin fungicide. An example of QoI fungicide resistance in the Dothidiomycete fungus class is in *C. sojina,* which causes frogeye leaf spot in soybean (Shrestha et al., 2017; Zhang et al., 2012). Shrestha and colleagues also made an interesting finding where they used a mixed mode of action fungicide containing a QoI group and a DMI group, and found an increased proportion of resistant *C. sojina* isolates, suggesting selection pressure for QoI resistance in mixed fungicides (Shrestha et al., 2017). In 2011 Bradley and Pedersen did a study on the baseline sensitivity of *C. zeae-maydis* to QoI fungicides, and they found that the fungicide concentration at which half of the conidial germination was inhibited ranged between 0.0003 – 0.0031 µg/ml depending on which QoI active ingredient was used (Bradley and Pedersen, 2011). The conidial germination of *C. zeae-maydis* was the least affected by the azoxystrobin group, showing more sensitivity to the pyraclostrobin and trifloxystrobin groups, showing that the QoI fungicide groups have different intrinsic activities and this may play a role in their sensitivity (Bradley and Pedersen, 2011).

One of the controversies of using a synthetic fungicide is the possible harmful effects on animals and humans. The QoI fungicides have shown low toxicity against mammals and birds, although the use of the QoI fungicides have been linked to toxicity to aquatic and amphibian animals including tadpoles and frogs, especially when high doses were applied (Johansson et al., 2006; Belden et al., 2010). The QoI fungicide class has shown to dissipate quickly in the environment making its accumulation and potential for long-term exposure quite low (Bartlett et al., 2002).

Demethylation inhibitor (DMI) fungicide group, which is also called the Sterol Biosynthesis Inhibiting (SBI) fungicides, are used against a range of foliar pathogens, including *C. zeina*. The DMI fungicides contains different classes of active ingredients, but their main function is to inhibit ergosterol biosynthesis by binding to the cytochrome P450 lanosterol demethylase and thereby disrupting the fungal membrane (Kuck et al., 2012). The DMI azole classes inhibits the methylation of the 14-α carbon

target enzyme, CYP51, found in the outer membrane of the endoplasmic reticulum, which is a member of the cytochrome P450 family. The oxidative demethylation of cytochrome P450 is important for the biosynthesis of ergosterol and other fungal sterols, enabling the fungicide to control fungal growth (Brent and Hollomon, 1995; Ma et al., 2006).

Continual and high usage of the DMI fungicides could also lead to resistance development, whereas this resistance development, unlike with the strobilurins, require multiple mutations (Luo et al., 2008). Mechanisms of DMI resistance include: (i) point mutations in the 14α-demethylase (*CYP51*) gene causing a decrease in affinity for the target gene (Asai et al., 1999; Delye et al., 1997; Ma et al., 2006); (ii) overexpression of the *CYP51* gene leading to increased number of target genes, and not enough fungicide to target all the gene copies (Hamamoto et al., 2000; Schnabel and Jones, 2001); as well as (iii) the overexpression of ATP-binding cassettes transporters which may affect the uptake or the efflux balance of the fungicide (de Waard, 1997; Gisi et al., 2000; Hayashi et al., 2002). Over the last few years certain *Fusarium* species have emerged with resistance against DMI fungicides, imploring the need for alternative management strategies (Koch et al., 2013; Yin et al., 2009). Recently *Alternaria alternate,* belonging to the Dothideomycete class and causing leaf blight in pistachio, has been found to be resistant against three different classes of DMI fungicides (Avenot et al., 2016; Yang et al., 2019).

Mixed mode of action fungicides which usually contain two or more active ingredients, as well as using a mixture of two different fungicides can be used to manage resistance development (van den Bosch et al., 2014). Certain aspects need to be taken into account when mixing two different fungicides including the effect of the dose of the two fungicides used on selection for resistance, the effectiveness of the fungicides after mixture, as well as the effect that two at-risk fungicides might have on resistance management (van den Bosch et al., 2014). Mixing different fungicide groups will not prevent resistance development completely, but this will help to slow the rate of spread of resistant mutant groups (Vincelli, 2002). Most mixed mode of action fungicides are a mixture of strobilurin and triazole fungicides which, if applied correctly, could sufficiently protect the crop against disease and decrease the potential of resistance development (Paul et al., 2011). However there has been some cases where cross-resistance between fungicides with different modes of action has been observed, including in *A. alternata,* as well as in *Pyrenophora tritici-repentis* (Deising et al., 2008; Malandrakis et al., 2015; Yang et al., 2019).

Some of the fungicides used against GLS in South-Africa include:

- Difenozim SC which is a systemic fungicide from Universal Crop Protection that uses the active ingredients, carbendazim (triazole) and difenoconazole (benzimidazole).
- Aroxy 250 SC from Arysta Life Sciences which is a broad-spectrum fungicide, and has systemic, translaminar and contact action and uses azoxystrobin (strobilurin) as the active ingredient which inhibits mitochondrial respiration.

- Abacus® Advanced from BASF SA is a broad spectrum, systemic fungicide with epoxiconazole as its active ingredient, which is a type of triazole, inhibiting fungal respiration in a site-specific manner.
- Nativo from Bayer which is a systemic fungicide uses tebuconazole (triazole) and trifloxystrobin (strobilurin) as the active ingredients.

Due to the high risk of resistance development against fungicides, as well as the harmful environmental effects of conventional fungicides, novel disease management strategies are necessary. Using RNAi as a type of disease management fungicide method could be beneficial as it is an environmentally friendly as well as a non-GMO approach.

2.2 RNA interference

RNAi machinery and mechanisms

RNA interference (RNAi) is a mechanism which describes the hybridization between RNA strands which is complementary to specific messenger RNA (mRNA) strands, leading to gene silencing, rendering the desired transcripts non-functional (Fire et al. 1998). This phenomenon was first described in the nematode *Caenorhabditis elegans* by Fire and his colleagues in 1998, describing RNAi as a biological silencing process in these organisms. It is proposed that the function of RNAi and similar processes are to protect organisms' genomes against harmful elements such as transposons and viruses, and that this mechanism is widespread in all eukaryotic organisms (Agrawal et al., 2003; Shabalina and Koonin, 2008; Casas-Mollano et al., 2016). RNAi has now been shown to form the basis for processes previously called co-suppression in plants, and quelling in fungi (see section on RNAi against fungi) (Agrawal et al., 2003; Eamens et al., 2008; Fulci and Macino, 2007).

The three key proteins which are involved in the RNAi pathway in eukaryotes, including plants and fungi, are the Argonaute-PIWI protein family, Dicer-like protein endonucleases, which contains a RNAseIII domain, and RNA-dependent RNA polymerase (RDRP) (Casas-Mollano et al., 2016; Shabalina and Koonin, 2008). Small non-coding RNAs (sRNA) which are approximately 20-25 base pairs in length, associate with these RNAi proteins to regulate gene expression and ultimately cause gene silencing (Carthew and Sontheimer, 2009; Guo et al., 2016).

The general process of RNA interference is graphically represented in figure 1.2. This process starts with either single-stranded RNA (ssRNA) being converted into double-stranded RNA (dsRNA) by an RNA-dependent RNA polymerase (RDRP) enzyme or exogenous dsRNA entering the cell via RNA viruses, transgenes or synthetically applied dsRNA (Agrawal et al., 2003; Fischer and James, 2004). The dsRNA is then recognized by the ribonuclease enzyme called Dicer, which will then cut up this

dsRNA into smaller 21-25 base pair small RNA (sRNA) pieces (Agrawal et al., 2003; Novina and Sharp, 2004). These sRNA pieces will then join the RNase complex called the RNA-induced silencing complex (RISC), containing the Argonaut (Ago) proteins which will cleave one of the strands leaving a single-stranded RNA piece (Agrawal et al., 2003; Novina and Sharp, 2004). The remaining RNA strand guided by the RISC complex, which includes proteins such as nucleases, will then bind to the complementary/partially complementary mRNA strand. This could potentially result in cleavage and degradation of the mRNA, rendering the gene non-functional (Agrawal et al., 2003; Majumdar et al., 2017).

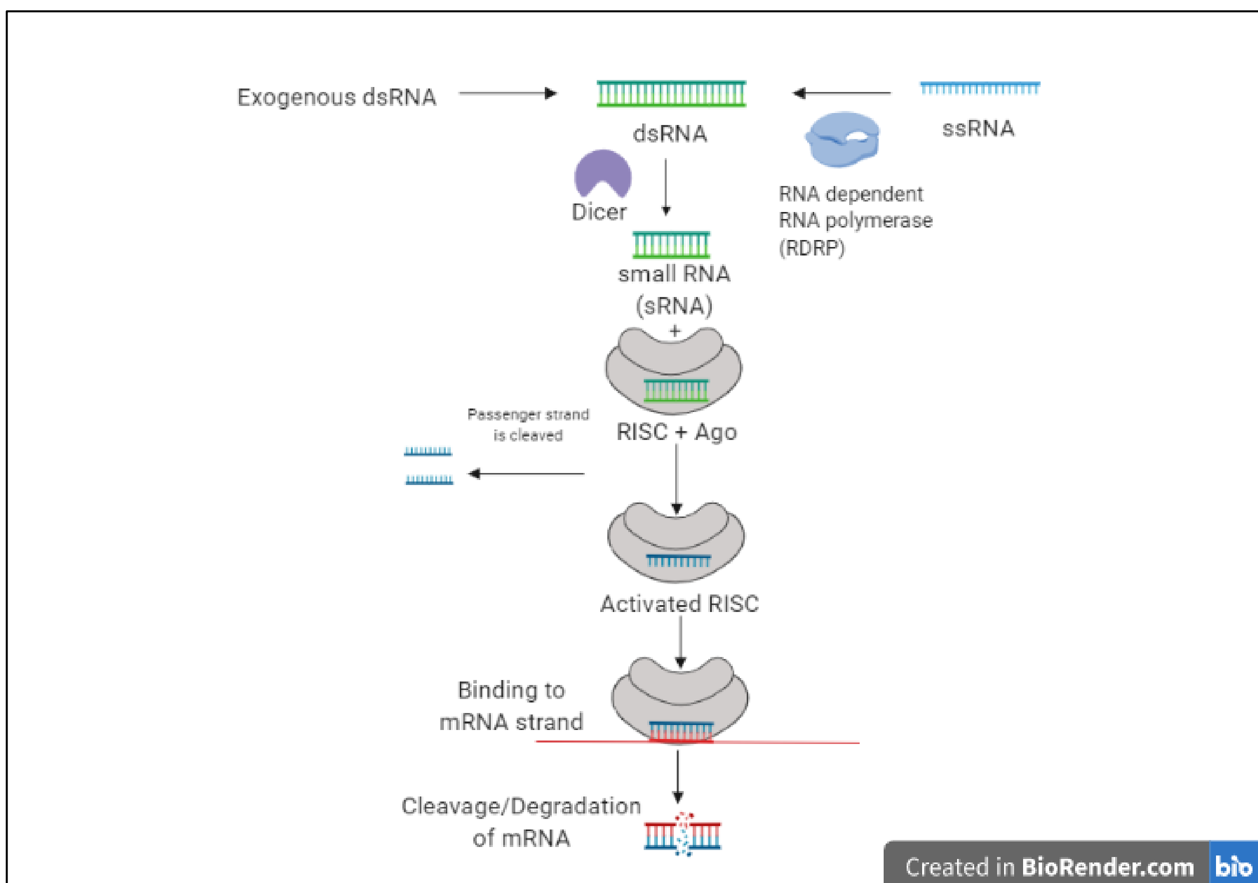


Figure 1.2: Graphical example of the general RNAi process. Firstly, the dsRNA is either transcribed by RNA-dependent RNA polymerases (RDRP), or it enters the organism from an exogenous source, this is recognized by the Dicer enzyme and cut into smaller pieces. The small RNA (sRNA) is then incorporated into the RNA-induced silencing complex (RISC) containing the Argonaute protein (Ago), and one of the strands is cleaved. The RISC then binds to the small RNA's complementary mRNA strand and cleaves the mRNA. Adapted from Majumdar et al. (2017).

Antisense RNAs are types of endogenous non-coding RNA which are used for regulating gene activity at different levels in cells by affecting chromosome structures, transcription or translation (Rusk, 2014). These antisense RNA molecules are small (about 19-23 bp), diffusible between cells, and they complement specific mRNA (Rusk, 2014; Xu et al., 2018). The two major types of antisense RNAs, also called small RNAs (sRNAs), involved in eukaryotic gene silencing are the small interfering RNAs (siRNAs) and the microRNAs (miRNAs) (Carthew and Sontheimer, 2009). These sRNAs play a role in various biological processes such as apoptosis, histone modifications, cell proliferation and cell growth (Bushati and Cohen, 2007; Wilson and Doudna, 2013). miRNAs have been shown to primarily play a role in regulating endogenous genes, whereas siRNAs respond to invading or foreign RNA, for example from virus infections, protecting the integrity of the organism's genome (Carthew and Sontheimer, 2009).

miRNAs are created from endogenous hairpin-like transcripts (Han et al., 2006). These transcripts get transcribed into pri-miRNAs by RNA polymerase II and this pri-miRNA is processed in the nucleus by a Drosha enzyme into pre-miRNA and exported into the cytoplasm via a nuclear transport factor (Cai et al., 2004; Lund et al., 2004). Once in the cytoplasm the pre-miRNA is cleaved by a Dicer enzyme to create the mature miRNA strand which then associates with effector proteins, creating a RNA-induced silencing complex (RISC), enabling gene silencing by binding with partial complementarity to mRNA of target genes (Lee et al., 2004; Schwarz et al., 2003).

siRNAs are formed from RNA-dependent RNA polymerases which are recruited to template RNA strands forming long dsRNA transcripts, which are then processed by Dicer in animals or Dicer-like in plants in the cytoplasm (Baulcombe, 2004; Molnar et al., 2010; Pinzón et al., 2019). siRNAs also associate with a RISC complex to ultimately induce silencing. Unlike miRNAs, the siRNAs do not require Drosha for processing and for effective silencing these siRNAs require binding to their target sequences with full complementarity (Fritz et al., 2006; Lee et al., 2004).

dsRNA was shown to be the initiator of gene silencing caused by RNAi in plants (Waterhouse et al., 1998). When both sense and anti-sense transgene sequence strands containing the potato virus Y (PVY) were hybridized to form a double stranded duplex and transfected into tobacco plants, they exhibited better silencing against the virus (better protection), compared to when both strands were used separately (Waterhouse et al. 1998). The expression of a hairpin RNA from a transgene in a transformed plant, has been shown to cause much more efficient gene silencing compared to using single stranded sense or anti-sense constructs alone, emphasizing the necessity of dsRNA in the RNAi silencing phenomenon (Waterhouse et al., 1998; Wesley et al., 2001).

Silencing signals are mobile and the silencing effect can move and spread systemically from the initial site of silencing to other parts of the organism (Palauqui et al., 1997). In *C. elegans* it was shown that systemic RNAi require the protein SID1, allowing passage of dsRNA through different cell membranes

(Feinberg and Hunter, 2003; Winston et al., 2002). sRNAs in animals move intercellularly, between adjacent cells, through gap junction structures (Lim et al., 2011; Valiunas et al., 2005). Plants do not use the same dsRNA transport system as found in nematodes and mammals, as there are no membranes which must be passed during the movement between plant cells. All plant cells are connected through plasmodesmata channels, thus it is believed that the silencing signal moves intercellularly through these plasmodesmata channels (Haywood et al., 2002; Molnar et al., 2011). The silencing signal in plants appears to move systemically within the phloem from source to sink tissues, whereas the xylem was shown to be free of RNA (Melnyk et al., 2011; van Bel, 2003). Investigating proteins in the phloem sap, responsible for the transport of sRNAs, researchers found a protein called the Phloem Small RNA Binding Protein1 (PSRP1) in plants such as pumpkin, cucumber and lupin (Yoo et al., 2004). It was found that these PSRP1 proteins could transfer 25-nucleotide single stranded RNA molecules between distant cells through the phloem sap, but it was not able to transport any double-stranded RNA molecules (Yoo et al., 2004).

RNA interference in fungi

The main components in the fungal RNAi system includes the Dicer-like proteins (DCL), the Argonaute (AGO) proteins and the RNA-dependent RNA polymerase (RdRP) proteins. The DCL proteins are part of the RNAseIII family and they process dsRNA into either siRNA or miRNA (Meng et al., 2017). The AGO proteins bind to the siRNAs or the miRNAs and form RNA-induced silencing complexes which will then facilitate gene silencing (Nguyen et al., 2018). The RdRP will produce dsRNA within the fungal organism, and cause silencing to be initiated (Bai et al., 2012).

Filamentous fungi has been shown to use their RNAi machinery mostly for post-transcriptional gene silencing (PTGS), rather than transcriptional regulation (Chicas et al., 2005; Freitag et al., 2004). Quelling is a phenomenon found in fungi caused by transgene-induced gene silencing, which is commonly referred to as RNA interference (Romano and Macino, 1992; Chang et al., 2012). This quelling phenomenon was discovered when researchers transformed a *N. crassa* strain with *albino* genes, involved in carotenoid biosynthesis. The transformants were a pale yellow colour, compared to the bright yellow wild-type fungal colony There was a reduction in the *albino* mRNA levels, indicating the silencing of the endogenous *albino* genes by the newly introduced transgenes (Cogoni et al., 1996; Fulci and Macino, 2007; Romano and Macino, 1992). Machino and Cogoni also isolated different *quelling-defective* (*qde*) mutants and did complementation studies with these genes. This showed that they encode for specific RNAi proteins including RNA-dependent RNA polymerase (QDE-1), Argonaute (QDE-2) and a helicase (QDE-3), indicating the importance of the QDE proteins in fungal RNAi (Cogoni and Macino, 1997; Dang et al., 2011). Neurospora Dicer-like proteins were also shown to play an important role in gene silencing, and when deleted, the quelling and Dicer activity was

impaired, indicating that quelling requires core RNAi components (Catalanotto et al., 2004; Chang et al., 2012).

Quelling occurs mainly in the vegetative tissue of filamentous fungi such as *N. crassa* and the process is mediated by specific small RNA molecules and plays a role in silencing homologous genes post-transcription (Cogoni and Macino, 1999; Yang et al., 2015). Tandem repeats of transgenes have been shown to be a trigger for the quelling process when *N. crassa* is growing under normal conditions (Lee et al., 2009; Wang et al., 2012). When the fungus is exposed to a DNA damaging agent another specific type of small RNA plays a role in gene silencing called qiRNA, which interacts specifically with the Argonaute protein, QDE-2 (Lee et al., 2009). The qiRNAs are a type of small RNA which is derived from repetitive sequences of ribosomal DNA, which is shorter than the normal sRNAs, and requires the same QDE and RNAi components as normal quelling (Lee et al. 2009). This qiRNA process is thought to play a role in the DNA damage response by inhibiting the translation of faulty proteins (Lee et al., 2009). The DNA damage induced sRNAs have also been observed in other eukaryotes including plants and mammals, indicating that this is a conserved mechanism across eukaryotes (Chen et al., 2013; Francia et al., 2012; Wei et al., 2012).

QDE-1 has been identified as a type of RNA-dependent RNA polymerase within *N. crassa* and plays a role in quelling (Cogoni and Macino, 1999). QDE-1 will convert aberrant single-stranded transgene transcripts into dsRNA which will subsequently be processed into smaller RNA products and induce transgene silencing (Forrest et al., 2004). The maintenance and rate of the silencing signal against foreign genes depend on the processing of RNA by QDE-1 (Forrest et al., 2004). When fungal cells directly express dsRNA or exogenous dsRNA enters into the fungal cells, the QDE-1 protein will no longer be necessary as the dsRNA will directly be recognized and further processed by the rest of the RNAi machinery (Catalanotto et al., 2004; Forrest et al., 2004).

QDE-2 found in *N. crassa* was shown to encode for an Argonaute protein which is similar to the Argonaute protein found in *C. elegans*, called RDE-1, and which is responsible for the dsRNA-induced silencing (Catalanotto et al., 2000). It is believed that post-transcriptional gene silencing induced by transgenes and RNAi share common genetic characteristics and possibly evolved from the same ancestral genes (Li et al., 2010). Quelling will happen during the vegetative life stage of the fungus and, thus quelling in fungi and RNAi in plants and animals are basically the same gene silencing mechanisms.

The model fungus, *N. crassa,* has also been shown to use its RNAi machinery for meiotic silencing by unpaired DNA (MSUD) (Shiu et al., 2001). MSUD happens during sexual development prophase I of meiosis when homologous DNA sequences which are not paired are detected and leads to the production of RNA transcripts which then leads to the silencing of the unpaired genes (Shiu et al., 2001). The MSUD silencing system required the Suppressor of *ascus* dominance 1 (*SAD1*) gene, which is

paralogous to the *QDE-1* gene, as well as DCL-1 and an AGO homolog. The QDE-2 protein and DCL-2 are not required for MSUD, thus in *N. crassa* there are two separate RNAi pathways which function at different life stages of the fungi.

Fungal RNAi pathways play a role in diverse processes including, fungal growth and development, pathogenicity, antiviral defense and protection of the genome (Meng et al., 2017; Wang et al., 2016; Weiberg et al., 2013). sRNAs has been shown to be responsive to a range of environmental stresses including in the fungi *M. oryzae* and *A. flavus* (Bai et al., 2015; Raman et al., 2017). In the fungus, *A. flavus*, miRNA-like sRNAs were shown to be expressed differentially at different temperature and water activity conditions, favouring the biosynthesis of mycotoxins (Bai et al., 2015).

miRNAs as explained earlier are endogenous non-coding RNAs, proven to play a role in gene silencing in plants, animals and algae (Billmyre et al., 2013). It was though that fungi did not possess such miRNA silencing pathways, but in 2010 it was discovered that *N. crassa* has miRNA-like sRNAs (milRNAs), describing four different classes (Lee et al., 2010). The fungal milRNAs are derived from single-stranded non-coding RNA transcripts which form hairpin structures (Lee et al., 2010). milRNAs has since been identified in a range of filamentous fungi including the plant pathogenic fungi, *S. sclerotiorum* and *Fusarium oxysporum*, and the entomopathogenic fungus *Metarhizium anisopliae* (Chen et al., 2014; Zhou et al., 2012). Some of the fungal milRNA pathways are Dicer independent, but they still depend on the QDE proteins. Overall, the milRNAs have been shown to work similarly to other miRNAs, such as animal siRNAs, by silencing endogenous mRNA transcripts with only partial complementarity (Lee et al., 2010; Billmyre et al. 2013).

RNAi machinery and mechanisms have been shown to be conserved in all the major fungal groups including Ascomycetes, Basidiomycetes and Zygomycetes (Cogoni and Macino, 1999; Nicolás et al., 2003; Wang et al., 2010). Despite the fact that RNAi is mostly conserved in the fungal kingdom, some fungal species have completely lost their RNAi functions. The model yeast *S. cerevisiae* has lost its RNAi pathway, but can be induced by introducing Dicer and Argonaute genes from the closely related species *S. castelli* (Drinnenberg et al., 2009). A fungal pathogen which has lost all of its RNAi ability is the maize yield limiting fungus *Ustilago maydis* (Billmyre et al. 2013). Most of the RNAi lost in fungi happened relatively recently, but the question remains why loss takes place (Drinnenberg et al., 2011). Some speculated reasons for RNAi loss in fungi could be due to the fact that certain fungi harbor viruses which could be advantageous to its survival by creating a competitive environment, thus RNAi which would normally inhibit the virus becomes non-functional (Drinnenberg et al. 2011). Another speculated reason for RNAi loss in fungi could be due to the fact that a fungus wants to adapt to a new environment quickly, thereby allowing transposon movement, creating a hypermutator phenotype (Billmyre et al., 2013; Oliver et al., 2000).

Certain fungi including Ascomycetes have hyphae that is separated by septa, and these septa contain pores (Wang and Dean, 2020). Studies regarding the movement of sRNAs between fungal tissue is mostly lacking, but transport of the sRNAs in fungi is speculated to be through septal pores and vesicles (Wang and Dean, 2020).

It has been proposed that RNAi in fungal pathogens plays a major role in their virulence. The fungus *Botrytis cinerea* was shown to express several sRNAs when infecting *Arabidopsis thaliana*, with these sRNAs playing a role in suppressing plant defense related genes and increasing the pathogenicity of the fungus (Weiberg et al., 2013). It is thus suggested that certain pathogen sRNAs can act similarly to effector proteins (Weiberg et al., 2013). Three core RNAi proteins were also reported to play a role in the pathogenicity of the fungus *Verticillium nonalfalfae,* including the AGO, DCL and RdRP proteins (Jeseničnik et al., 2019). Certain RNAi proteins are also believed to be important in growth and regulatory processes, with the Dicer-like proteins in *B. cinerea* shown to be important in vegetative growth as well as playing a role in processing sRNAs (Weiberg et al., 2013).

Different RNAi components within the *F. graminearum* fungus was analyzed for their role in sexual maturation, pathogenicity and if they perceive and process dsRNA, making them essential for exogenous RNAi (Gaffar et al., 2019). RNAi key components involved in fungal pathogenicity, specifically *F. graminearum*, were shown to be DCL2 and AGO1 (Chen et al., 2015). These two proteins also showed importance in silencing hairpin RNA, which could indicate that they also play a role in exogenous dsRNA silencing. An *F. graminearum* mutant for both DCL1 and DCL2 showed no reaction to externally applied dsRNA, indicating the importance of these genes for environmental RNAi (Gaffar et al., 2019)

A wide range of Ascomycete fungi exhibit a sex induced RNAi mechanism which could be called sex-specifically induced exonic small interference RNA (ex-siRNA) (Son et al., 2017). The ex-siRNA is used specifically in the sexual development stage. As with *N. crassa* the filamentous fungus *F. graminearum* contains the RNAi pathway used for sexual development and primarily uses DCL1 and AGO2 (Son et al., 2017). RdRP and a QDE-3 ortholog were later also shown to contribute to sexual reproduction within *F. graminearum*, but further transcriptomic data is necessary to explain the mechanisms (Gaffar et al., 2019). A range of milRNAs which possibly play a role in ex-siRNA mediated RNAi have been identified within *F. graminearum* and this process has been shown important in the development of ascospores (Zeng et al., 2018).

RNAi machinery in Dothideomycete fungi

A bioinformatics approach was taken in this study to characterize the RNAi machinery in *C. zeina* and Dothidiomycete fungi in general. Orthologs to three *N. crassa* RNAi machinery proteins, namely Dicer-like 1 (GenBank ID: XP_961898.1; Neucr2_5838), Dicer-like 2 (GenBank ID: XP_963538.3;

Neucr2_3123), and QDE-2 (GenBank ID: AAF43641.1; Neucr2_8697 and Neucr2_8698), a type of Argonaute, were identified within 99 Dothideomycete fungal genomes (Haridas et al., 2020) and outgroup fungi including two Eurotiomycetes, two Leotiomycetes, one Sordariomycete and one Pezizomycete genomes using Orthofinder v2;3;1 (Emms and Kelly, 2019) with an inflation factor of 2.5. Identified orthologous proteins were functionally annotated by GO (Blast2GO) and PFAM (Interproscan) analyses (Figure S1.1, S1.2 and S1.3). Orthologous proteins identified by Orthorfinder for each RNAi machinery protein were further curated by removing those that were significantly smaller in size compared to that of the *N. crassa* reference proteins. The *N. crassa* Dicer-like 1 (DCL-1) protein gene is 1584 amino acids in size, thus any orthologous proteins smaller than 1200 aa were removed. The *N. crassa* Dicer-like 2 (DCL-2) protein is 1396 amino acids in size, and any orthologous proteins smaller than 1100 were removed. The QDE-2 proteins are within the range of 1070 to 1090 amino acids and any proteins smaller than 900 amino acids were ignored. Additionally, GOs and PFAM domains were also compared to the reference proteins and those that did not have the same GO and PFAM domains with that of the reference were also removed. The curated protein dataset for each RNAi machinery protein was aligned using Muscle v. 3.8.31. Phylogenetic analyses were conducted using RAxML v.8.2.1 using 20 maximum likelihood searches and auto bootstrapping until convergence.

The outgroup fungal groups chosen within this study are fungi in which numerous RNAi-related studies were conducted. These fungi were from different fungal classes including, *F. graminearum,* which is characterized as a Sordariomyctes, *S. sclerotiorum* and *B. cinerea* which are characterized as Leotiomycetes, *Aspergillus nidulans* which is a Eurotiomyctes and *Tuber melanosporum*, a Pezizomycete. The RNAi orthologs from the different representative fungal species were aligned together with the *N. crassa* RNAi proteins, shown in Figures S1.1-S1.3, and indicate that certain domains within these proteins are conserved between fungal classes. *Cercospora zeina* was included in this alignment and it was shown that *C. zeina* also contains conserved regions for these three RNAi proteins (Figure S1.1-S1.3).

The *DCL-1* and *DCL-2* in *N. crassa* are homologous genes, and their proteins essentially perform the same function (Catalanotto et al., 2004). Both of these Dicer-like genes contain the four essential domains which is found in the Dicer protein family including two RNAse III domains, a RNA helicase domain and a DEAD-box ATP binding domain (Catalanotto et al., 2004). DCL-1 is a protein with 1584 amino acid sequences, whereas the DCL-2 protein consists of 1396 amino acids. A difference between the two proteins is that the DCL-2 contains an extra double-stranded RNA binding domain which is not present within the DCL-1 homolog (Catalanotto et al., 2004).

RNA-dependent RNA polymerase (RdRP) was not included in the ortholog analysis. The main reason was that the focus of the MSc dissertation was on dsRNA-mediated RNAi in fungi, and the RdRP is not expected to play a role when RNA in double-stranded form is taken up by fungal cells.

DCL-1 (Dicer Like-1 orthologs)

The *N. crassa* DCL-1 protein is a type of RNAseIII enzyme which processes dsRNA into smaller RNA nucleotides in an ATP-dependent manner and also plays a role in the sexual development of the fungus (Chang et al., 2012). The *N. crassa* genome contains a single copy of the *DCL-1* gene which codes for a 1584 aa protein. The phylogenetic data from DCL-1 is summarized in table 1.1 where it is shown that *C. zeina* clustered together with the Capnoidales class and had 1 copy of the protein, similar to *N. crassa* (Figure 1.3). Interestingly *Zymoseptoria pseudotritici* had no orthologs for DCL-1. *Bimuria novae-zelandiae* was the only fungus that contained more than one copy of DCL-1 (Table 1.1 and Figure 1.3).

DCL-2 (Dicer Like-2 orthologs)

The *N. crassa* DCL-2 protein also belongs to the RNAseIII enzyme family and cleaves dsRNA into sRNA, facilitating the RNAi process (Chang et al., 2012). The *N. crassa* genome contains a single copy of the *DCL-2* gene which encodes for a 1396 aa protein. *C. zeina* clustered with the Capnoidales fungal class and contained one copy of the DCL-2 protein, similar to *N. crassa* (Table 1.1 and Figure S1.4). There were several fungi which contained no orthologs to DCL-2, including the fungus, *Z. pseudotritici* and *Zymoseptoria tritici* (Table 1.1).

QDE-2 (Argonaute orthologs)

The *N. crassa* QDE-2 protein which plays a role in post-transcriptional gene silencing is a type of Argonaute protein (Li et al., 2010). The *N. crassa* genome contains two copies of this gene, both encoding proteins which are 1070-1090 aa in length. Most species, including *N. crassa* have 2 copies of the QDE-2 protein, and in most cases, these two copies clustered into two clades (Figure S1.5). *C. zeina* contained both copies of QDE-2, whereas *C. zeae-maydis* only contained a single QDE-2 copy. *Z. pseudotritici* contained no orthologous copy for QDE-2, similar to what was seen with DCL-1 and DCL-2 (Table 1.1). The fungus, *B. novae-zelandiae* had six copies of the QDE-2 protein.

Table 1.1: Summary of phylogenetic data on orthologs to *Neurospora crassa* proteins

N. crassa protein	Number of copies in *N. crassa*	Important protein domains	Capnodiales cluster[1]	*C. zeina* number of copies	Dothideomycete species containing no orthologs/less orthologs than expected	Species with more copies of orthologs than *N. crassa*[2]
DCL-1	1	-RNAse III activity domain -ATP helicase binding domain -siRNA production domain.	Normal clustering (*C. zeina* is within this cluster)	1	*Zymoseptoria pseudotritici*	*Bimuria novae-zelandiae* (2)
DCL-2	1	-RNA processing domain -RNAse III activity domain	Normal clustering (*C. zeina* is within this cluster)	1	*Dothistroma septosporum,* *Zymoseptoria tritici* *Hortaea acidophila* *Diplodia seriata* *Neofusicoccum parvum* *Macrophomina phaseolina* *Polychaeton citri* *Westerdykella ornate* *Zymoseptoria pseudotritici*	*Bimuria novae-zelandiae* (2) *Periconia macrospinosa* (2) *Plenodomus tracheiphilus* (2) *Pseudovirgaria hyperparasitica* (2) *Verruconis gallopava* (2)
QDE-2	2	-nucleic acid binding -protein binding -gene silencing.	Normal cluster (two different clades for the two copies. Each copy of *C. zeina* clustered within each clade)	2	*Bipolaris victoriae* *Bipolaris zeicola* *Cercospora zeae-maydis* (1) *Myriangium duriaei* *Zymoseptoria pseudotritici*	*Alternaria alternata* (4) *Bimuria novae-zelandiae* (6) *Botryosphaeria dothidea* (5) *Cucurbitaria berberidis* (4) *Lophiotrema nucula* (5) *Lophium mytilinum* (5) *Periconia macrospinosa* (5)

[1] Fungal order of Capnodiales clustering together according to known phylogenetic assumptions (i.e., Normal cluster - all Capnodiales do cluster together)

[2.] Number of gene copies for each species is shown in brackets () (i.e., all fungi indicated contain more gene copies than the *N. crassa* fungal species)

Loss and gain of RNAi

The loss in certain fungal RNAi pathways are believed to have occurred relatively recently (Drinnenberg et al., 2011). Losing the RNAi pathway could have brought some advantages in certain species of fungi. For example in the case of *Saccharomyces cerevisiae* losing the RNAi pathway has allowed it to house a dsRNA virus, also called the killer virus, which encode for a killer toxin that helps it outcompete other yeast species in the environment (Drinnenberg et al., 2011). The RNAi function in some fungi could have caused a specific disadvantage, and thus fungal genomes evolved to include RNAi-deficiency. Some fungi use retrotransposon rearrangement for increased virulence and thus a loss in functional RNAi pathway, will cause an increase in transposon activity, and increased fungal pathogenicity (Nunes et al., 2011; Wang et al., 2010). Some fungi are relatively devoid of transposable elements all together, thus there is no need to degrade harmful transposable elements, another possible reason for fungi to have lost their RNAi capabilities (Drinnenberg et al., 2009; Kämper et al., 2006).

Ustilago maydis, from the Basidiomycete class, previously described to have lost its RNAi functionality, also showed no orthologs to the rest of the RNAi genes, complementing previous findings that there are no AGO and DCL homologs within *U. maydis* (Kämper et al., 2006). The possible reason for the loss in RNAi capabilities within *U. maydis* is that it has very little transposable elements, whereas its close relative, *Ustilago hordei,* contains numerous transposable elements and has a functional RNAi system (Kämper et al., 2006; Laurie et al., 2008). This can probably be the same for the Dothideomycete fungi, where certain species could have lost their ability to silence genes via RNAi due to a lack of transposable elements within the genome. The fact that *U. maydis* lost its RNAi silencing capability, but its close relative, *U. hordei* still has a functioning RNAi pathway, could also mean that the loss of an RNAi pathway within fungi happens rarely and randomly.

The hemi-biotrophic fungus *Zymoseptoria pseudotritici*, which is closely related to *Z. tritici* had no orthologs to any of the RNAi proteins analyzed. This is interesting as the other two closely related *Zymoseptoria* spp. both had orthologs to some of the RNAi components. *Z. pseudotritici* has been classified as a hybrid species that evolved recently through interspecific hybridization of closely related species (Stukenbrock et al., 2012). During the hybridization event these RNAi machinery genes could have been lost as an evolutionary advantage. It is also possible that some errors occurred during the annotation of the genome of this fungus, causing these proteins not to be recognized.

Interestingly, the wheat fungal pathogen *Z. tritici* had no orthologs to the DCL-2 gene. The DCL-2 gene, unlike the DCL-1 gene, does not play a role in sexual maturation, but rather helps to silence hairpin RNA, and thus makes this important component in fungal pathogenicity. *Z. tritici* has been tested for its ability to utilise the HIGS strategy for disease and growth decrease, but this strategy was shown to be unsuccessful (Kettles et al., 2019). They also tested if *Z. tritici* was able to take up exogenous dsRNA, but this too was shown to be unsuccessful (Kettles et al., 2019). The small RNAs

within this fungus is processed through a Dicer-independent manner. A reason for the HIGS strategy not working in this fungus could be that the hairpin RNA does not get processed into smaller RNA due to the lack of DCL-2, hindering the whole gene silencing process. Some functional RNAi components are present within *Z. tritici*, but it is hypothesised that they can either be redundant or only play a small role during stress conditions (Kettles et al., 2019).

In this study the fungus, *Myriangium duriaei,* belonging to the Myriangiales order, only had an ortholog to the DCL-2 gene. No other RNAi ortholog was detected, indicating to a possible gene loss for DCL-1 and QDE-2, which may cause RNAi pathway to be insufficient. The *M. duriaei* fungus is a parasitic fungus which is found on scale insects. It would be interesting to look at possible reasons for this gene loss, starting with an analysis of the genome of this fungus, looking for any lack in transposable elements, or if they retain a killer virus which could increase its survival and competition within niche environments.

Dicer-like genes within plants were shown to undergo diversification through alternative splicing, however within fungi there haven't been enough evidence of diversification through alternative splicing, aside from a few fungi containing more than one gene copy (Choi et al., 2014; Margis et al., 2006). In this study a few fungi contained more than one copy of their DCL encoding genes, indicating that these genes play an important role within the fungal lifestyle. The fungus *Valsa mali* has been shown to utilise its Dicer-like genes during stress responses against hydrogen peroxide and calcium chloride, possibly indicating that fungi which is exposed to stressful environments on a regular basis evolved this DCL stress response, increasing their DCL gene copies (Feng et al., 2017).

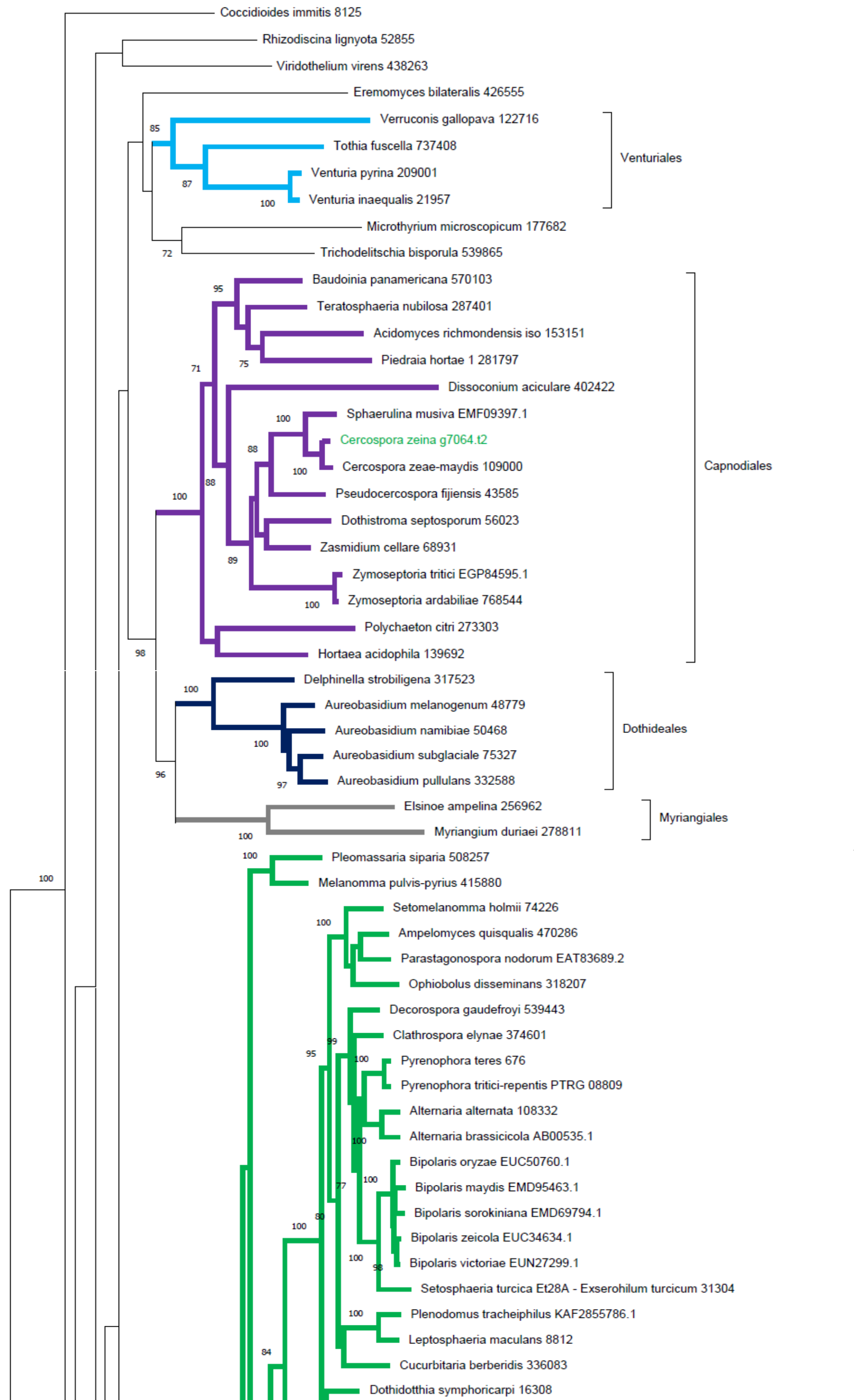
Cercospora zeina contains one DCL-1 ortholog, one DCL-2 ortholog, and two QDE-2 orthologs. This indicates that *C. zeina* has a functional RNAi pathway and could be used in RNAi-related studies. The multiple copies of QDE-2 within fungal species did not group together closely within their taxonomic clades, following the trend of most species seen on the tree (Figure S1.5), indicating that this gene duplication happened a long time ago, possibly even before the emergence of the Dothideomyctes. Within plants it is believed that the diversification of the DCL genes happened before the plants diverged into monocots and dicots, and the same could be true within fungi when looking at the Argonaute genes, possibly forming duplications before the fungi diverged into the different taxonomic classes (Choi et al., 2014).

QDE-2 has been shown to play a role in Dicer-independent processing of small RNAs, including specific milRNAs (Lee et al., 2010). The *QDE-2* gene had multiple copies within each species, indicating either functional diversification or functional redundancy, possibly due to its role in Dicer-independent sRNA processing. Previously, the RNAi components of *Verticillium nonalfalfae* were analysed and they also identified two copies of QDE-2 which were not closely related in sequence or length (Jeseničnik et al., 2019). A previous study also found that the majority of ascomycete fungi

belonging to the fungal subphylum Pezizomycotina, contained two or more copies of QDE-2 (Choi et al., 2014).

The fungus, *Bimuria novae-zelandiae* was first isolated from soil in a barley field, in New Zealand and is thought to be a saprophyte (Hawksworth et al., 1979). *Bimuria novae-zelandiae* is the only fungus which had more than two copies of both the *DCL* genes as well as 6 copies for the *QDE-2* gene. This fungus showed the most gain in RNAi machinery from all the different fungal species. The reason for this gain in function could be that the genome contains numerous transposable elements, or this fungal species is prone to viral attack and the RNAi is a defence mechanism. Little is known about this fungus, and it would be interesting to assess if this RNAi mechanism evolved to help with the survival of the fungus or as an aid to the pathogenesis of the fungus.

A few unknowns still exist as to why certain fungi harbour extra copies of the RNAi genes compared to others that completely lost all of their RNAi genes. The RNAi machinery genes do not only play a role in genome protection, but they have very diverse roles within fungal life styles, including sexual maturation, conidiation, fungal development, pathogenicity and antiviral defence (Gaffar et al., 2019). It would be interesting to further link all of these genes to specific functions and pathways within different fungi and uncover why certain fungi lack these genes.



Botci1 13594
Coccidioides immitis 8125
Rhizodiscina lignyota 52855
Viridothelium virens 438263
Eremomyces bilateralis 426555
Verruconis gallopava 122716
Tothia fuscella 737408
Venturia pyrina 209001
Venturia inaequalis 21957
Venturiales
Microthyrium microscopicum 177682
Trichodelitschia bisporula 539865
Baudoinia panamericana 570103
Teratosphaeria nubilosa 287401
Acidomyces richmondensis iso 153151
Piedraia hortae 1 281797
Dissoconium aciculare 402422
Sphaerulina musiva EMF09397.1
Cercospora zeina g7064.t2
Cercospora zeae-maydis 109000
Pseudocercospora fijiensis 43585
Dothistroma septosporum 56023
Zasmidium cellare 68931
Zymoseptoria tritici EGP84595.1
Zymoseptoria ardabiliae 768544
Polychaeton citri 273303
Hortaea acidophila 139692
Capnodiales
Delphinella strobiligena 317523
Aureobasidium melanogenum 48779
Aureobasidium namibiae 50468
Aureobasidium subglaciale 75327
Aureobasidium pullulans 332588
Dothideales
Elsinoe ampelina 256962
Myriangium duriaei 278811
Myriangiales
Pleomassaria siparia 508257
Melanomma pulvis-pyrius 415880
Setomelanomma holmii 74226
Ampelomyces quisqualis 470286
Parastagonospora nodorum EAT83689.2
Ophiobolus disseminans 318207
Decorospora gaudefroyi 539443
Clathrospora elynae 374601
Pyrenophora teres 676
Pyrenophora tritici-repentis PTRG 08809
Alternaria alternata 108332
Alternaria brassicicola AB00535.1
Bipolaris oryzae EUC50760.1
Bipolaris maydis EMD95463.1
Bipolaris sorokiniana EMD69794.1
Bipolaris zeicola EUC34634.1
Bipolaris victoriae EUN27299.1
Setosphaeria turcica Et28A - Exserohilum turcicum 31304
Plenodomus tracheiphilus KAF2855786.1
Leptosphaeria maculans 8812
Cucurbitaria berberidis 336083
Dothidotthia symphoricarpi 16308

Figure 1.3: DCL-1 Phylogenetic tree. RAxML tree constructed in this study from DCL-1 orthologs from species of Dothideomycetes, Leotiomycetes, Eurotiomycetes and Sordariomycetes. The tree was constructed using the Maximum Likelihood method based on 20 searched. Bootstrapping was done until convergence and values are shown at the nodes. *Neurospora crassa* the reference fungus is shown in red and *Cercospora zeina* which had a single DCL-1 ortholog is shown in green.

Cross-kingdom RNAi

Small RNAs moving between organisms and causing gene silencing, called cross-kingdom RNAi, has been shown between different organisms such as plant and animal hosts and their interacting pathogens and parasites (Wang et al., 2017). This process along with the HIGS process is illustrated in figure 1.4, where sRNAs are shown to move between a pathogenic fungus and the plant host. The fungal pathogen *Botrytis cinerea* has developed a virulence mechanism against host plants using cross-kingdom RNAi (Weiberg et al., 2013). *B. cinerea* sRNAs enters into its host plant and will silence specific host immunity genes such as the mitogen-activated protein kinases (MAPKs) and cell wall-associated kinases (WAKs) genes, increasing the pathogenic virulence (Weiberg et al., 2013).

Plants have also been shown to export certain miRNAs onto infecting pathogens, for example cotton plants infected with *Verticillium dahliae* causing the devastating Verticillium wilt disease responded by producing defense miRNAs which could enter the pathogen and protect the plants (Zhang et al., 2016). The RNA silencing role in the pathogenic strategy of the *V. dahliae* fungus was investigated by deep sequencing sRNAs from hyphae recovered from infected cotton plants, and 28 of the sRNAs identified were similar in sequence to cotton miRNAs (Zhang et al. 2016). The cotton plants have adapted to *V. dahliae* pathogenic infection by exporting miR166 and miR159, which was most abundant, to the fungal hyphae for silencing certain genes important for fungal virulence, such as Ca^{2+}-dependent cysteine proteases (Zhang et al. 2016).

RNAi can vary across species in the duration of the silencing effect, as well as the systemic spread of the silencing signal (Zotti et al., 2018). The spread of the silencing signal between cells and tissues could lead to the dsRNA reaching the germline cells of the organisms and this signal being passed on to the progeny of the next generation (Zotti et al. 2018). The systemic spread of the silencing signal suggests that there is a mobile signal specific to silencing associated with the RNA silencing pathway which is not yet known in most organisms (Mlotshwa et al., 2002).

The RNAi machinery in plants have evolved to target viruses with RNA genomes, using siRNAs, and it has been shown that certain host-specific RNAi gene expression is induced when a virus infection occurs (Bai et al. 2012). Cucumber Mosaic virus infection enhanced RNA-dependent RNA polymerase genes in *Salvia miltiorrhiza* (Red sage) (Shao and Lu, 2014). Tomato yellow leaf curl virus (TYLCV) infection was shown to play a role in increased expression of DCL and Ago genes in tomato plants (Bai et al. 2012; Shao and Lu 2014; Muhammad et al. 2019). The RNAi components DCL2 and DCL4 have been shown to be crucial in protecting plants against invading viruses, whereas the plants with inactive DCL2 and DCL4 had much higher virus accumulation (Andika et al., 2015; Dzianott et al., 2012).

Plant survival depends on the effectiveness of its defense strategies, such as the siRNA-mediated gene silencing against invading viral infections (Moon and Park, 2016). This is an advantageous strategy as

this silencing signal can spread throughout the plant (Moon and Park 2016). However, this siRNA-mediated defense strategy is only effective after a virus has invaded the host plant and might be unable to resist invading viruses (Gan et al., 2008). miRNAs can exist within a plant cell before a viral infection and because of the binding ability of miRNAs, binding only with partial complementarity to target sequences, the binding and silencing efficiency is increased, expanding the target spectrum and increasing the plants defense capability (Gan et al., 2008; Liu et al., 2017).

Pathogens, such as well-adapted plant viruses, have however also evolved a virulence strategy to overcome host plant gene silencing by triggering certain suppressors which targets host RNAi genes (Muhammad et al., 2019). The cucumber mosaic virus (CMV) has evolved a suppressor protein called the 2b protein which drastically reduces the siRNAs produced by three different Dicer genes in *Arabidopsis* (Diaz-Pendon et al., 2007). When a CMV 2b-deletion mutant was inoculated onto *Arabidopsis* plants the amount of viral siRNAs was higher compared to inoculations with the wild type CMV (Diaz-Pendon et al., 2007).

Until recently it was unknown how plants send their sRNAs into infecting pathogens. Host plant cells transferring their endogenous sRNAs to pathogens were thought to be a selective process and not only a concentration gradient diffusion process (Cai et al., 2018; Wang and Dean, 2020). In animal cells, extracellular vesicles were shown to play a role in systemic transport of sRNAs and it was thought that similar vesicles are used by plants (Mittelbrunn and Sánchez-Madrid, 2012). Host sRNAs were identified in vesicle libraries and these sRNAs were shown to be protected from nucleases, confirming that the transferred sRNAs are inside vesicles and not bound to the outside surfaces (Cai et al., 2018). The vesicle hypothesis was proven in 2018 by Cai et al., by showing that the model plant *Arabidopsis* delivers its sRNAs into the fungus *Botrytis cinerea* by using extracellular vesicles resembling exosomes.

2.3 Applications of RNAi in different organisms

Understanding Gene function

The RNA-based silencing phenomenon has been proven to silence expression of specific genes, rendering this mechanism a good candidate for studying gene and protein function. siRNA can be used to knock down mRNA and protein expression in cultured cells, delineating the role of the specific regulatory proteins on gene expression (Curtis and Nardulli, 2009). The RNAi process can be used to identify synergies and signaling pathway interactions, but it also allows researchers to find the roles of functionally redundant genes by targeting multiple genes at the same time (Fraser, 2004; Gotta and Ahringer, 2001; Ooi et al., 2003)

RNAi can be used successfully within a number of insect/pest and nematode species, revealing functions of proteins and also the response of different life stages to gene silencing. Systemic RNAi effects on the red flour beetle, *Tribolium castaneum* has been shown, where GFP dsRNA was injected into the beetle larval cavity and was shown to inhibit GFP expression through development of pupal and adult stages (Tomoyasu and Denell, 2004). Inducible RNA interference in *Drosophila melanogaster* revealed the physiological roles of different proteins which are involved in the mitochondrial metabolism (Matsushima et al., 2007). RNAi was also used to study the role of a gene which encodes the novel parasitic nematode-specific protein (*SDNP*) in *Setaria digitata* larvae, indicating that this SDNP plays a role in larvae development processes, parasitism and muscle contraction (Somarathne et al., 2018).

The first discovery of RNAi playing a role in immune responses was in shown in plants which showed increased immunity when infected with viruses (Baulcombe, 2004; Navarro et al., 2006; Nimchuk et al., 2003). Tobacco plants which were infected with tomato black ring nepovirus underwent transgene-induced gene silencing by producing certain RNAi components that protected the plant against the virus and caused a decrease in viral symptom development (Ratcliff et al., 1997). Shortly thereafter, plants were shown to also exhibit RNAi immune responses against bacterial infections (Baulcombe, 2004; Navarro et al., 2006). In 2006 Navarro et al., showed that *Arabidopsis* plants infected with *Pseudomonas syringae*, produced certain plant miRNAs which are induced by the bacterial antigen, flg22, and in turn down-regulates certain host susceptible genes, aiding in the plant's anti-bacterial resistance.

RNAi used in therapeutics in Medicine

RNA interference usage in therapies such as cancer is an ongoing and exciting field of study. Complementary target siRNAs can be applied directly to cells or tissues, or siRNAs can be expressed by hairpin structures, targeting genes of interest for degradation (Davidson and McCray Jr, 2011). Some of the barriers which may hinder the use of RNAi as therapeutic agents include: (i) the RNAi system must have a definite specificity to the target gene; (ii) delivering the RNA to the correct cell or tissue might be an issue; (iii) the longevity and resilience of the RNAi activity (Davidson and McCray Jr 2011).

Some advances which have been made to enable the efficient delivery of siRNAs as therapeutic agents include using lipid nanoparticles as well as siRNA conjugates (Kanasty et al., 2013). Unmodified siRNAs are unstable and are unable to cross membranes and enter different cells, thus chemical modifications or other delivery materials are needed to transport such siRNAs to their desired sites of action (Kanasty et al., 2013; Whitehead et al., 2009). An example of a successful delivery system is where siRNA is conjugated to lipophilic molecules which in turn interact with serum lipoproteins and will then enter into hepatocytes through the uptake of these lipid proteins (Wolfrum et al., 2007).

Host-induced gene silencing

Host-induced gene silencing (HIGS) is a RNAi-based process where certain sRNAs are expressed in plant hosts which would then in turn silence pest and pathogen genes (Nowara et al. 2010). The HIGS process between plants and pathogenic fungi is shown in figure 1.4. This works by expressing the desired dsRNA in transgenic plants where the sRNAs will be processed and used for specific silencing. One of the advantages of this plant protection strategy is the specificity with which it can target genes for silencing, limiting off-target undesired effects. A disadvantage to the HIGS strategy is the requirement of plants to be transformed, thus a specific transformation strategy for the host plant needs to be established. Another disadvantage is the strict regulations which apply when approving the GMO HIGS-based plant products for commercialization.

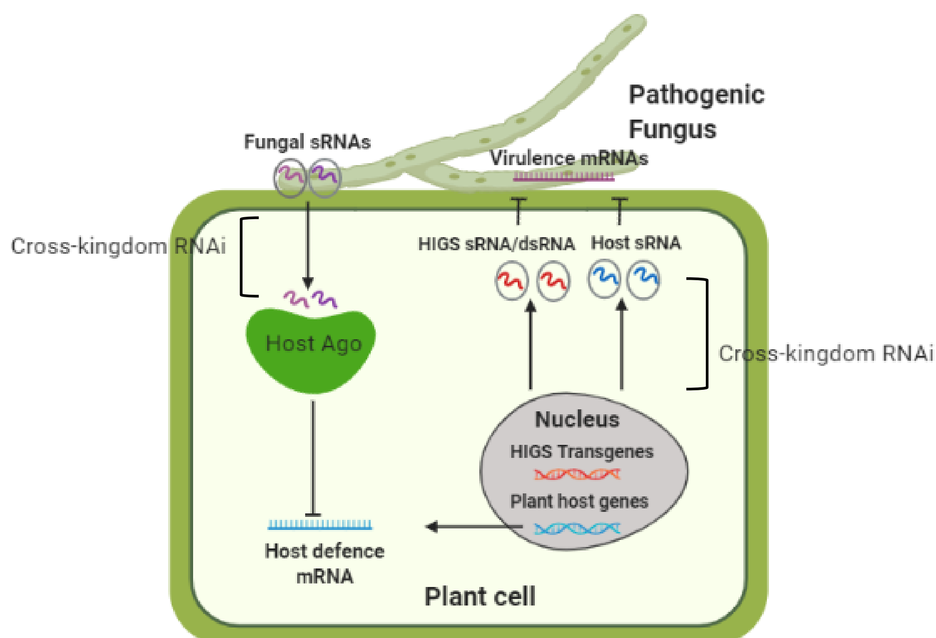


Figure 1.4: Graphical representation of both cross-kingdom interactions between a plant cell and a pathogenic fungus as well as the phenomenon, Host-induced gene silencing (HIGS). Small RNAs (sRNAs) produced by the plant can cross between the plant and the fungus and silence specific fungal virulence genes. With HIGS, transgenic plants express double-stranded or hairpin transgenes which get processed and transported to the pathogenic fungus. Similarly, fungal sRNAs can cross between the fungus into the plant and silence specific host defense mRNAs, using the hosts own RNAi mechanism. Adapted from Weiberg et al (2015).

HIGS has been used successfully against plant pests, such as the western corn rootworm, where transgenic maize plants expressed dsRNA targeting the vacuolar ATPase genes, caused a reduction in feeding damage caused by these worms (Baum et al., 2007). Another example of the success of HIGS-based strategy against plant pests is where cotton plants were engineered to express the cytochrome P450 dsRNA, CYP6AE14, increasing resistance to bollworm (Mao et al., 2007). HIGS has also been used to protect soybeans from the *Meloidogyne incognita* nematode, by causing a reduction in root gall formations between 90-95% (Ibrahim et al., 2011).

HIGS strategies have also been shown effective in protecting transgenic plants from fungal infections. Transgenic barley and wheat were made expressing dsRNA targeted against the *Blumeria graminis Avra10* effector gene, which helps to support the establishment of the disease in host plants (Nowara et al., 2010). A significant decrease in powdery mildew infection was observed on these transgenic dsRNA-expressing barley and wheat plants, indicating the success of the HIGS strategy against the *B. graminis* pathogen infection (Nowara et al. 2010). Recently a HIGS system expressed in wheat against the devastating stripe rust pathogen *Puccinia striiformis* f. sp. *tritici* was successful in targeting a Protein kinase A pathogenicity factor called PsCPK1, leading to the reduction in the length of infection hyphae and disease phenotype (Qi et al., 2019; Qi et al., 2018).

HIGS has also been shown to be effective against the necrotrophic pathogen *S. sclerotiorum* and the oomycete *Phytophthora capsici*. The *S. sclerotiorum* chitin synthase gene was targeted using the HIGS strategy and a reduction in disease severity of 50-80% was observed (Andrade et al., 2016). A *P. capsici* avirulence gene was targeted by expressing a dsRNA complementary to the a-virulence gene within the host plant tissue (Vega-Arreguín et al., 2014). A downregulation in avirulence gene expression was observed after the pathogen was inoculated onto the transgenic hairpin-producing plants (Vega-Arreguín et al., 2014).

Plant parasites such as the Orobanchaceae species which parasitizes the roots of host plants, utilising the host plant water and nutrients, could have a huge negative impact on the world's agriculture., *Cuscuta pentagona*, the parasitic dodder plant showed disruption in dodder growth when grown together with tobacco plants created through HIGS targeting a known dodder functional gene (Alakonya et al., 2012). Transgenic lettuce roots expressing hairpin RNA complementary to specific GUS genes was grown along with the transgenic *Triphysaria versicolor* parasite, expressing this GUS gene (Tomilov et al., 2008). The steady state levels of GUS mRNA were analysed and a reduction in mRNA levels was seen where the parasite interacted with this transgenic lettuce. A bidirectional movement of the silencing signal between parasitic plant and a second plant host was also observed, indicating that the silencing signal can be moved to additional hosts using the parasite as a physiological bridge. The silencing of parasitic genes using this HIGS strategy could be used in future as a novel strategy to control such parasitic pests.

External RNAi application

Due to previous work, it is clear that dsRNA plays a very important part in natural RNAi, thus the HIGS concept was used to produce dsRNA within the host as a control mechanism. Double-stranded RNA (dsRNA) occurs naturally in most organisms in nature, triggering RNAi which plays a role in antiviral immunity (Ding, 2010). This has led scientists to explore if the external application of dsRNA could overcome the limitations associated with HIGS control strategies, for example eliminating the need to transform the host plant. As dsRNA is a naturally occurring product, it could be an advantageous, environmentally friendly mechanism of controlling pests and diseases, replacing conventional and harmful chemical pesticides, herbicides and fungicides. Another advantage of applying dsRNA instead of chemical compounds is the fact that the dsRNA mechanism relies on specific nucleotide sequences as targets and do not act in a structurally dependent manner, rendering it much more target specific.

Producing long dsRNA constructs may be advantageous as they can be used as a pyramidal approach to plant protection in which the dsRNA which has homology to a range of diverse pathogenic targets, can be fused and may confer simultaneous protection against pathogens in one host plant (Niehl et al., 2018). An added advantage to dsRNA is that the gene silencing mode of action of the dsRNA works throughout the entire sequence length, making pathogen resistance through sequence mutations highly unlikely. dsRNAs are also biodegradable and occur inside eukaryotic organisms, the environment and even food (Niehl et al., 2018). Certain companies synthesize dsRNA products for various uses, one such company is called AgroRNA, a subdivision of Genolution Inc. which is based in Seoul, Korea.

Using dsRNA technologies on a large scale such as greenhouse or field trials depends on the quality of the dsRNA, the stability as well as efficient mass production (Mitter et al., 2017; Niehl et al., 2018). In 2018, Niehl et al. described an efficient dsRNA production system in the bacterium, *Pseudomonas syringae,* which relies on the replication of the dsRNA using RNA-dependent RNA polymerase. RNase-deficient *E. coli* systems has also been shown to produce large scale dsRNAs where hairpin expression vectors were made containing T7 promoter sequences and expressed in RNAseIII-deficient *E. coli* where after dsRNA was eluted by nuclease digestion with RNAses (Thammasorn et al., 2015).

Naked dsRNA could be unstable when sprayed directly onto plant surfaces, or applied to pests such as Lepidoptera insects, and become unable to confer protection for long periods of time, increasing the need to create ways of delivering stable dsRNAs (Terenius et al., 2011). dsRNAs are unfortunately unstable in water and soil environments. dsRNA was undetected two days after being added onto soil, regardless of different soil conditions (Dubelman et al., 2014). In 2017, Mitter et al., showed that dsRNA could be loaded onto a non-toxic, degradable, layered double hydroxide (LDH) clay nanosheet, which helps to stabilize the dsRNA used against specific plant viruses for up to 30 days (Mitter et al., 2017). dsRNA which target chitin synthase genes in mosquitos were coated onto nanoparticles

containing the polymer, chitosan, and fed to mosquito larvae. The larvae showed between 30 to 60% reduction in chitin synthesis (Zhang et al., 2010). Another novel stable way of delivering dsRNA to plants could be by forming a complex with synthetic dsRNA and a carrier peptide (Numata et al., 2014). Coating of naked dsRNA onto specifically designed nanoparticles aid the stability and effectiveness of the dsRNA and could thus be used for commercializing dsRNA compounds against pests and plant diseases.

External RNA applied against fungal pathogens

Fungal cells, such as *B. cinerea* and *F. graminearum* have been shown to successfully take up externally applied sRNAs and dsRNA from the environment (Koch et al., 2016; Wang et al., 2016). This discovery could lead to the development of external RNAi-based disease control strategies, one of these strategies include SIGS which stands for Spray Induced Gene Silencing, which entails dsRNA targeting pathogenic genes being sprayed directly onto the host (Wang and Jin, 2017). Table 1.2 indicates the different fungal pathogens targeted using exogenous dsRNA, and the percentage decrease in gene activity as well as pathogenicity observed. In 2016, Wang et al. showed that *B. cinerea* spores and protoplasts were able to take up externally applied fluorescently labelled dsRNAs, shown in figure 1.5. dsRNA was designed to be complementary to certain *B. cinerea* Dicer-like genes, which are important for pathogenicity of the fungus, and a reduction of the BcDcl-1/2 gene expression was observed. This concept was also tested *in planta* on fruits such as strawberries, vegetables such as lettuce and even rose petals, showing a reduction in lesion size and fungal biomass after the RNA inoculations (Wang et al., 2016). This study forms the basis for using RNAi to silence fungal pathogenicity genes and ultimately protect host plants from yield limiting fungal diseases.

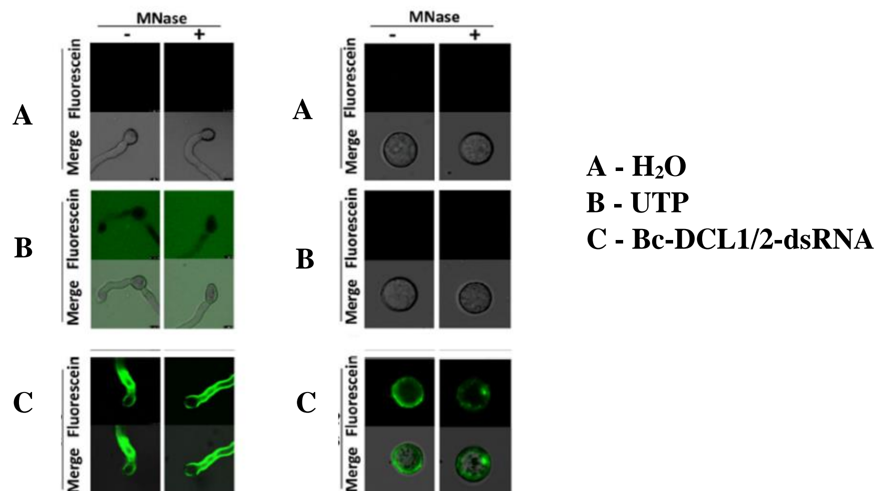


Figure 1.5: ***Botrytis cinerea*** **spores and protoplasts show uptake of fluorescently labelled dsRNA.** Fluorescein- UTP (neg control), water (neg control) and fluorescently labelled Bc-DCL1/2 were added onto both *B. cinerea* protoplasts and spores. Micrococcal nuclease (MNase) was added to wash away any unbound RNA, and it is clear that the labelled dsRNA entered into the fungal cells compared to the negative controls. Copied from Wang et al. (2016).

In 2016, Koch et al., designed long non-coding dsRNAs which was complementary to *F. graminearum* ergosterol biosynthesis genes, such as the *CYP51* genes, and after applying the RNA to barley leaves and then infecting the leaves with the pathogen, less severe pathogenic lesions were observed as shown in figure 1.6a. Figure 1.6b indicates the decrease in amount of fungal DNA after the CYP3-dsRNA has been added compared to GFP and Tris-EDTA controls, showing significantly lower levels than the controls (Koch et al., 2016). Figure 1.6c shows the expression levels of the three specific ergosterol genes compared to the GFP control, where a significant reduction in expression for these three genes can be seen. Recently it has also been shown that other *Fusarium* species are susceptible to uptake and silencing of ergosterol genes by this similar *CYP51*-derived RNA, adding to the potential of this novel plant defense strategy (Koch et al., 2018).

Sclerotinia sclerotiorum is a devastating disease responsible for white stem rot disease which causes great crop losses worldwide on a wide range of crop hosts including canola, soybean and sunflower (Kamal et al., 2016). Genetic control of this pathogen has been ineffective and traditional agrochemical control of the pathogen colonization has also been not been as effective (McLoughlin et al., 2018). In 2018, McLoughlin et al. studied the effect of external dsRNA targeted against a wide range of genes, for example reactive oxygen species genes and transcription factor genes, identified through differential expression analysis of the *S. sclerotiorum* pathogen both *in vitro* and *in planta* on resistant and susceptible *Brassica napus* strains. In the *in vitro* cultures, the transcript levels were reduced between 48-59% after 48 hours of incubation with the dsRNA. Gene ontology (GO) term enrichment analysis identified 59 potential dsRNA targets (McLoughlin et al., 2018). During the *in planta* tests where *B. napus* was infected with the pathogen, 20 out of the 59 dsRNA targets used showed a reduction in lesion size, making them good targets for RNAi (McLoughlin et al., 2018).

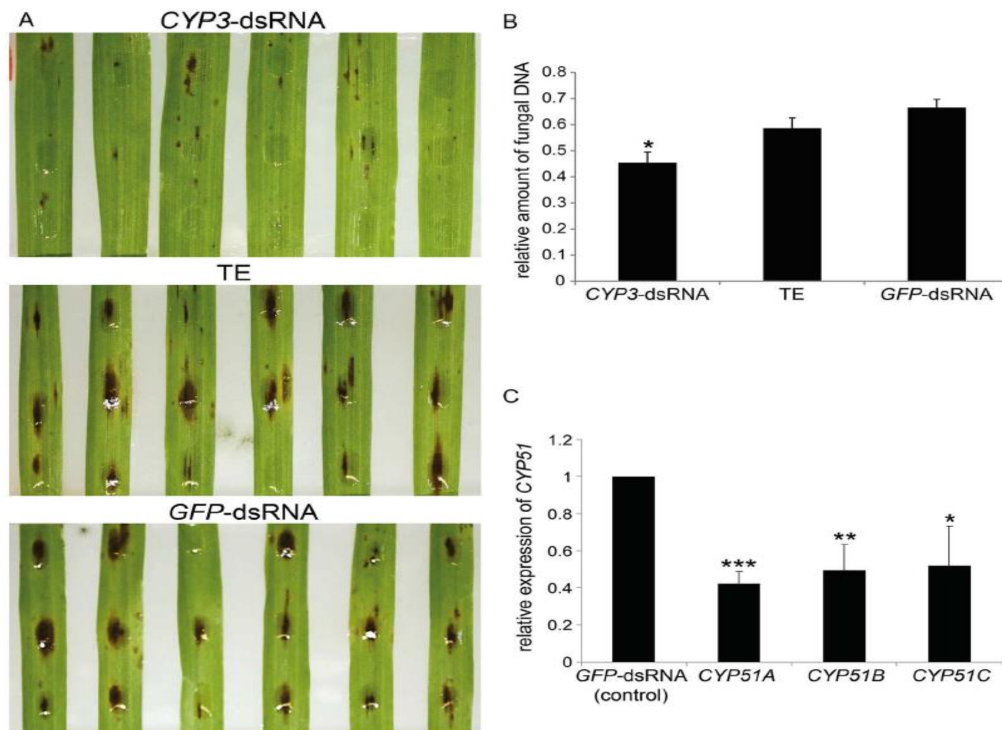


Figure 1.6 Results of SIGS treatment against *Fusarium graminearum*. A, Addition of CYP3-dsRNA to *F. graminearum* infected barley leaves. After first adding the CYP3-dsRNA and then infecting barley leaves with *F. graminearum*, a decrease in the lesion severity can clearly be seen when compared to the two controls, TE and GFP-dsRNA. B, The relative amount of fungal DNA on the dsRNA treated leaves. The amount of fungal DNA after the addition of the CYP3-dsRNA is significantly lower than the amount of fungal DNA when compared to the control treated leaves. C, The relative expression of the target genes. The three specific target gene expressions were all significantly lower when compared to the GFP-dsRNA control gene expression. Adapted from Koch et al. (2016)

RNA sequencing technologies were used successfully in the past to identify good targets for the RNAi mechanisms, looking at pathogenic genes which are upregulated during infection processes and also genes which are specific to the pathogen and that does not have conserved orthologous within the plant host (McLoughlin et al., 2018). Potential targets for RNAi could be pathogen virulence factors, effector genes or certain pathogen essential genes, including examples such as the protein which forms a cap binding complex which is involved in the mRNA translation process, translation initiation factors proteins, eIF4, or also important transcription factors.

Recently in 2019, Gu et al. suggested a new anti-fungal agent which uses *β_2-tubilin*-derived dsRNA from *Fusarium asiaticum* against a diverse range of pathogens. The *β_2-tubilin* gene was chosen in this RNAi protection strategy as a target, due to this gene being conserved among fungal species and thus could be used to control a variety of pathogenic species without inducing resistant or tolerant strains (Gu et al., 2019). Wheat, barley and cucumber infected with *F. asiaticum*, *M. oryzae* and *B. cinerea* respectively were sprayed with the *β_2-tubilin* dsRNA and showed a significant decrease in disease

symptoms when compared to controls and a significant decrease in the amount of fungal DNA was also observed (Gu et al., 2019).

RNAi technologies has also been used to reduce allergenic proteins in foods such as carrots (Koch and Kogel, 2014). RNAi-mediated the knockdown of two allergenic proteins, Dau c 1.01 and Dau c1.02, creating carrot lines with only minor allergenic reactivity in allergic patients (Peters et al., 2011). These proteins are part of the pathogenesis-related 10 (PR10) plant protein family and it remains uncertain whether the silencing of these genes may have a negative impact on the defense ability of the plant against pathogens.

It has been shown that unlike with HIGS, where the plant's RNAi machinery is necessary for producing siRNAs and causing gene silencing, SIGS require the fungal RNAi machinery for causing gene silencing (Gaffar et al., 2019; Koch et al., 2016). Specific RNAi machinery fungal mutants, where the DCL1 and DCL2 genes were not working, were tested for their compatibility with SIGS, and were shown to be compromised in their SIGS activity (Gaffar et al., 2019; Koch et al., 2016).

SIGS-mediated fungal inhibition has been shown to be independent of plant immune responses. Externally applied dsRNA onto barley leaves infected with *F. graminearum* showed no elicitation of the plant's immune response called, pattern triggered immunity (PTI) (Koch et al., 2016). Thus, SIGS does not activate any host defense pathways.

RNA-based targeting of fungal genes is not always that successful as it depends on the RNA silencing machinery of the fungus as well as the ability of the fungus to take up RNA. One example of where both HIGS attempts and RNA uptake has been shown to be unsuccessful is in the fungus *Zymoseptoria tritici* (Kettles et al., 2019). The authors suspected that the reason for the unsuccessful uptake of the dsRNA and unsuccessful HIGS attempt could be due to the fact that the *Z. tritici* RNAi pathway only plays a small role during the growth of the fungus or plays a role only during stressed conditions (Kettles et al., 2019). Certain fungal species including, the pathogen *Ustilago maydis,* has lost all of its RNAi functionality, making it unable to utilise the RNAi protection strategy (Billmyre et al., 2013).

Table 1.2: Summary of pathogenic fungal genes targeted by external dsRNA

Fungus	Host plant	Genes targeted	Protein Function	Size of dsRNA	Concentration of dsRNA	% gene expression decreased	% decrease in disease	Reference
Botrytis cinerea	Fruits and vegetables	DCL1/2	RNAi machinery, growth and development	490 bp	400 ng/µl	Up to 70%	Up to 80%	Wang et al. 2016
Fusarium graminearum	Barley	CYP51	Ergosterol biosynthesis.	791 bp	1000 µg/ml	Up to 60%	Up to 50%	Koch et al. 2016
Sclerotinia sclerotiorum	*Brassica napus*	Wide range	Growth/developmen t and pathogenicity	200-450 bp	100-1000 ng/ml	Up to 50%	Up to 80%	McLoughlin et al. 2018
Fusarium graminearum	Barley	AGO and DCL	RNAi machinery, growth and development	1500-1800 bp	1000 µg/ml	Up to 70%	Up to 60%	Werner et al. 2020

External RNA applied against pests

Pests such as the Western corn rootworm (WCR) has been shown to ingest dsRNA produced by transgenic maize, an example of cross-kingdom movement of RNA (Baum et al., 2007). The maize was transformed to express dsRNA against the insect V-ATPase A gene which is an important regulator of proton movement in insect epithelia (O'Donnell, 2017; Wieczorek et al., 2009). A higher rootworm mortality and less feeding damage was observed with the transgenic maize, suggesting that the desired dsRNA from the maize was ingested orally by the WCR, ultimately showing that cross-kingdom interference between species is possible (Baum et al., 2007).

Different agricultural pests have been shown to respond differently to RNAi mechanisms. Coleoptera pests have been shown to process dsRNA which has been obtained from oral feeding on transgenic plants, whereas Lepidopteran pests has shown little response towards dsRNA. A comparison was made between the core RNAi machinery of the Western corn rootworm (WCR), the fall army worm (FAW) and the southern green stink bug (SGSB) (Davis-Vogel et al., 2018). It was hypothesised that a variation in the natural RNAi characteristics could cause a variation in efficacy of pesticidal RNA. The core RNAi machinery was found to be a Drosha protein, and two Dicer protein genes with one homologue per pest for each of these genes (Davis-Vogel et al., 2018). Different Argonaute 1 and 2 isoforms were identified in the different insect groups, whereas the expression pattern of the RNAi protein machinery was found to be similar in all three insects (Davis-Vogel et al., 2018). These studies on the different responses of insects to environmental dsRNA could form the basis for further studies on the mechanisms and interactions of the core RNAi machinery of each of these pests.

dsRNA was shown to be a stable insecticide when sprayed against the Colorado potato beetle, *Leptinotarsa decemlineata*, when the dsRNA-actin was applied onto host potato plants (San Miguel and Scott, 2016). The non-foliar, root application of dsRNA was also shown to be effective in causing mortality in both brown planthoppers and Asian corn borers (Li et al., 2015).

Environmental RNAi has not been successful in all cases, with the success relying largely on the type of organisms targeted, the target genes and the method of RNA delivery. Certain crop pests in the orders Lepidoptera and Hemiptera have been shown to be unresponsive to environmental RNAi, implying that certain biological barriers will limit the use of RNAi against insect pests (Baum and Roberts, 2014; Terenius et al., 2011). Certain insects contain specific double-stranded ribonucleases inside their lumen which could digest the dsRNA and cause them to be trapped inside acidic bodies, hindering the dsRNA to be processed into siRNAs (Yoon et al., 2017). New ways in which such barriers could be overcome needs to be investigated, for example the use of certain specifically designed nanoparticles to deliver the dsRNA successfully.

2.4 Conclusion

As we have a constant growing global population, the demand for food increases drastically. Unfortunately, the crop yields which would ideally be achieved are not possible due to external factors such as environmental changes and attack by pests and diseases. While environmental changes are quite hard to overcome, we have the technology to help fight against biotic factors, using fungicide and pesticide sprays. While there are a range of fungicides on the market, the continuous usage of such fungicides cause resistance development and can therefore not be used on a long-term basis. Most fungicides used also have adverse effects on non-target organisms as their mode of action targets a general pathway found in many organisms. This has led to the need for alternatives to fungicide sprays and the research into RNAi-based control approaches. This new field of study could open up new ways of safely protecting crops from pests and diseases, by either targeting one specific pest/disease at a time or targeting a range of different pests/diseases using one application and having little to no off-target effects.

The aim of this study is to identify *C. zeina* genes which can be targeted by a novel RNAi approach, create a double-stranded construct targeting these genes and apply this to the fungus. The hypothesis is that the fungus will take up the RNA and process it, and then use it to silence the specific target genes, ultimately decreasing the expression of the genes, and thereby lowering the pathogenicity of the fungus. This study lays the foundation for the development of a novel RNAi fungicide targeting yield limiting cereal pathogens.

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Chapter 2

Research chapter: External RNA interference against the fungus *Cercospora zeina*

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This chapter has been prepared in the format of a manuscript for a research journal (Fungal Biology). Dr. B.G. Crampton contributed to the original conceptualization of the project. Prof. D.K. Berger supported me with the strategy and design of the project. Prof. J. Theron provided valuable input regarding the synthesis and use of the double stranded RNA. I conducted all of the experiments, interpreted the data and put the manuscript together. Prof. D.K. Berger and Prof. J. Theron critically reviewed the manuscript and made valuable suggestions regarding the interpretation and presentation of the data.

Abstract

External RNA uptake and interference is a new emerging field of disease control which has shown great potential against fungal diseases such as *Botrytis cinerea* and *Fusarium graminearum*. This study aimed to test the novel RNA interference (RNAi) application against the fungus *Cercospora zeina,* responsible for causing grey leaf spot disease on maize. Management practices against this disease are not always effective and that is why it is important for other methods of control to be explored. In this study we showed that *C. zeina* mycelia, spores and protoplasts were able to take up externally applied fluorescently labelled double-stranded RNAs (dsRNAs). Transgenic *C. zeina* expressing GFP was treated with dsRNA targeting the GFP gene. GFP protein fluorescence was decreased by as much as 50%. A dsRNA construct was created in which three *C. zeina* genes were targeted namely, the chitin synthase D (*ChsD*) gene, the phosphatidylserine decarboxylase proenzyme 3 (*Psd3*) gene and the extracellular protein 2 effector gene (*Ecp2*). After *in vitro* inoculation of the fungus with the dsRNA construct, expression of the *ChsD* gene was significantly downregulated by 50 %. Cell viability of the fungus after application of the dsRNA construct was tested using a colorimetric assay, called an MTT assay, and a significant decrease of 34 % in cell metabolic activity was observed compared with control treated samples. The results obtained from this study form the basis to further develop RNA-based fungicides which target certain metabolic and pathogenic genes, which are expressed within the *C. zeina* fungus, when infecting its host plant, and represents a novel non-genetically modified (non-GM) disease management strategy.

Keywords: RNA interference, *Cercospora zeina*, Grey leaf spot disease, dsRNA, fungal infection

1. Introduction

The production of maize, a global staple food crop, is threatened by a variety of pests and diseases, one of them being the foliar fungal pathogen *Cercospora zeina*, which causes the grey leaf spot disease (GLS) (Crous et al., 2006; Goodwin et al., 2001; Meisel et al., 2009; Wang et al., 1998). This pathogen belongs to the dothideomycete class, which contains a variety of important plant pathogens including, *Cladosporium fulvum,* the devastating tomato pathogen (Ohm et al., 2012). GLS causes necrotic lesions on the maize leaf blade leading to major yield losses where in severe cases up to 65% loss was reported (Meisel et al., 2009; Ward, 1996). *C. zeina* spores overwinter within the soil and when favourable environmental conditions such as relative high temperatures together with high humidity occurs, the spores will germinate and start to infect the host maize plants (Meisel et al., 2009; Ward et al., 1999).

GLS is mainly managed using strobilurin fungicides, also called Quinone outside inhibitor (QoI) fungicides, which act by reducing the respiration capacity of the fungus (Bradley and Pedersen, 2011; Fernández-Ortuño et al., 2006, 2008). Certain *Cercospora sojani* and *Cercospora beticola* strains

showed enhanced resistance against QoI fungicides due to the high volume of fungicide spray application (Karadag and Yavuz, 2010; Zhang et al., 2012). *Cercospora zeae-maydis* has been tested on its baseline sensitivity to different QoI fungicides and it has been shown that *C. zeae-maydis* is able to use alternative respiration pathways to overcome the inhibitory effect of these fungicides (Bradley and Pedersen, 2011). *Cercospora zeae-maydis* and *C. zeina* has not shown fungicide resistance within the field as the spray regimes are closely monitored, however if spray applications are increased there is a high chance for resistance development (Bradley and Pedersen, 2011). Due to the nature of resistance development of pathogenic fungi against most fungicides, there is an increasing need for novel ways to control fungal diseases.

RNA interference (RNAi) is a phenomenon which has first been characterized within the nematode *Caenorhabditis elegans*. RNAi was shown to be a biological silencing process which protects the organisms against harmful elements such as viruses (Agrawal et al., 2003; Fire et al., 1998). The RNAi process has been utilized in applications such as Host-induced gene silencing (HIGS), where specific small RNAs (sRNAs) are expressed within a plant host to silence harmful pest and pathogen genes, acting as a control method (Nowara et al., 2010; Qi et al., 2019). The problem with using HIGS as a disease control method is that it uses genetic modification, making commercialization of products more difficult and time consuming.

Recently, external RNAi, a novel way of treating pests and diseases on plants has been shown to be a promising control strategy. Certain pests such as the Colorado potato beetle and the xylem-feeding leafhopper showed increased mortality rates after external treatment of host plants with double-stranded RNA (dsRNA) (Hunter et al., 2012; San Miguel and Scott, 2016). Fungal pathogens such as *Fusarium graminearum*, *Botrytis cinerea* and *Sclerotinia sclerotium* have also previously been treated with external RNA while infecting their plant hosts, and showed a decrease in disease severity (Koch et al., 2016; McLoughlin et al., 2018; Wang et al., 2016). This disease control strategy has also been termed spray induced gene silencing (SIGS), and has been proposed as a sustainable and environmentally friendly disease control strategy (Wang and Jin, 2017).

In this study we assessed if *C. zeina* fungal cells were able to take up externally applied dsRNA. We also tested if the RNA could be used to knockdown GFP protein fluorescence in a transgenic GFP-expressing *C. zeina* strain. *C. zeina*-specific genes were targeted by a dsRNA construct for disruption of gene function and the expression of each of these genes were analysed. The fungal metabolic activity of *C. zeina* spores targeted by dsRNA was also assessed after treatment with dsRNA.

2. Material and methods

2.1 Fungal and plant materials

Cercospora zeina isolate nr: CMW 25467 (Meisel et al., 2009) (Referred to a WT-*C. zeina*) as well as the *C. zeina CzmCTB7* transformant expressing the GFP gene (Obtained from Velushka Swart, University of Pretoria (Swart et al., 2017)) (Referred to as GFP-*C. zeina*), was cultured on solid V8 media (Beckman and Payne, 1982). The *CzmCTB7* transformant was created previously by *Agrobacterium* transformation of WT-*C. zeina* with the *Cercospora zeae-maydis ctb7* expression cassette as well as a GFP expression cassette. The V8 media was supplemented with 50 µg/ml of Cefotaxime. Cultures were kept in the dark at room temperature and sub-cultured weekly.

2.2 Synthesis of RNA constructs

All primers used in this study are listed in Table S2.1. A dsRNA construct targeting the GFP gene was created through PCR amplification of a 365 bp fragment within the GFP gene, with the primers containing T7 overhangs. These fragments were then transcribed into either fluorescent dsRNA using the Fluorescein RNA mix (Roche Applied Science, Germany) or into non-fluorescently labelled dsRNA using the HighScribe T7 in-vitro transcription kit (New England Biolabs, Massachusetts, USA). The PCR reaction was set up as follows: GFP specific primers were added at a final concentration of 10 mM, pJet 1.2_gpdAsGFP plasmid was used as template (10 ng/µl), and 1X Taq DNA polymerase RED (Amplicon, Denmark) was included in the reaction. Thermo-cycling reaction was run on a 2720 Thermal Cycler (Applied Biosystems, California, USA) as follows: Initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of 30 seconds at 95 °C, 30 seconds 59 °C, 30 seconds 72 °C, and the final extension was done at 72 °C for 2 minutes.

All dsRNA products were purified after transcription using the NucleoSpin RNA kit (Macharey-Nagel, Pennsylvania, USA). The dsRNA was heated at 94 °C for 5 minutes and left at room temperature to reanneal before electrophoresing it on the agarose gel and before storage. This heating step was done to help remove any secondary structure formation.

An RNA construct which targeted three *C. zeina* genes were generated and was called Cz3_dsRNA. *C. zeina* gene targets were chosen on the following criteria: (i) Genes with a function in either pathogenicity or cell viability; (ii) genes conserved in *Cercospora* species as well as genes that are also present in other pathogenic species; (ii) Genes reported for previous use in gene knockdown studies; (iii) Genes with unrelated sequences to maize – BLAST protein sequence to B73 RefGen_v4 maize genome using tBLASTn; (iv) Genes that were expressed in planta with a log2 counts per million (CPM) greater than two (GEO dataset: GSE94442); (v) Relatively high gene expression was desired *in vitro* on corn meal agar and V8 media (GEO dataset: GSE90705); (vi) Gene sequences

which were not be duplicated in the genome – BLAST to *C. zeina* genome PacBio v 2.0 assembly. Data for genes chosen is shown in Table S2.5 and S2.6.

C. zeina gDNA extraction was done as previously described, using the CTAB extraction method (Muller et al., 2016; Nsibo et al., 2019) and the gDNA was used as template for the following PCR reactions. The three genes code for the following proteins, respectively: Chitin synthase D (CHSD) (Genbank accession number: PKS02949.1), Phosphatidyl serine decarboxylase proenzyme 3 (PSD3) (Genbank accession number: PKS00696.1) and Effector protein 2 (ECP2) (Genbank accession number: PKR94769.1). *C. zeina* gene annotations were obtained from the genome sequence (GenBank accession: MVDW00000000) (Wingfield et al., 2017). Expression data used for gene selection were obtained from GEO datasets GSE90705 (*in vitro*) and GSE94442 (*in planta*) (Swart et al., 2017). This construct was created by PCR amplifying a region of each gene separately from the gDNA (obtained as described earlier), and then amplifying them together using an overlap PCR method (Figure S2.7) (Heckman and Pease, 2007). The PCR reactions were set up as follows: different primer pairs at a final concentration of 10 mM were mixed with *C. zeina* Mkushi gDNA at a final concentration of 40 ng/µl together with 1X Taq DNA polymerase RED (Amplicon). The reactions were incubated in a thermocycler at the following conditions: denaturation for 3 minutes at 95 °C, elongations for 25 cycles at 30 seconds 95 °C, 30 seconds 60 °C and 30 seconds 72 °C, and annealing for 3 minutes at 72 °C.

This final PCR product containing segments from each of the three genes (Figure S2.7) was then cloned into the pJet1.2/blunt (Figure S2.8) vector using the CloneJet kit (Thermo-Fisher Scientific, Massachusetts, USA). The final 1.4 kb product within the plasmid was sequenced to make sure the whole product, containing regions targeting each of the three genes mentioned above, was cloned into the vector (Figure S2.9). The 1.4 kb product was then amplified from the plasmid using a regular PCR and the product was extracted from a 1% agarose gel using the Zymoclean™ Gel DNA recovery kit (Zymo Research, Irvine, USA). The dsRNA was transcribed from the gel-extracted 1.4 kb DNA product using the HighScibe T7 in-vitro transcription kit (New England Biolabs) following the manufacturer's instructions (final concentration of DNA template used ~600 ng/µl).

2.3 In vitro *fungal RNA inoculation and analysis*

The *C. zeina* protoplast extraction protocol was adapted from the *Huntiella* species protocol (Wilson et al., 2020). *C. zeina* conidia which were growing for 6 days on solid V8 media were washed with ddH$_2$O, isolating the spores. The spore culture was incubated in YEPD media for 30 hours at room temperature, with gentle shaking. The mycelia were collected by filtering the liquid culture through a single layer of sterilized Miracloth® (Sigma-Aldrich, Missouri, USA) and resuspended in 1.2 M KCl. Protoplasts were created by adding the mycelia to lysing buffer (500 mg *Trichoderma harzianum* lysing enzyme (Sigma-

Aldrich), 25 ml 1.2 M KCl), for 14 hours. The protoplasts were harvested through centrifugation at 4000 rpm for 5 minutes and resuspended in a 1X STC (20 % sucrose, 50 mM Tris pH 8, 50 mM $CaCl_2$) solution.

The fluorescent dsRNA was incubated with three forms of *C. zeina* namely, conidia, mycelia and protoplasts. Fluorescent dsRNA at a final concentration of 374.8 ng/µl was added to 10 µl of $1x10^6$ protoplast ml^{-1} suspended in 1X STC buffer. Fluorescent dsRNA at a final concentration of 937 ng/µl was incubated with 8 µl of $1x10^6$ spores ml^{-1} *C. zeina* conidia and 8 µl of *C. zeina* mycelia suspended in YEPD media for 14 hours. Fluorescein-12-uridine-5´-triphosphate (Fluorecein-12-UTP) (Sigma-Aldrich) and ddH_2O were used as negative controls. RNA uptake was observed using a LSM 880 confocal microscope (Zeiss, Oberkochen, Germany) at the Laboratory for Microscopy and Microanalysis of the University of Pretoria. Excitation and emission wavelengths were set to GFP and Fluorescein-12-UTP standard wavelengths. Images were created and analysed using ZEN 2 software (blue edition) (Zeiss).

Non-fluorescent dsRNA targeting the GFP gene (GFP_dsRNA) was added at different concentrations (0.3 µM, 0.9 µM and 2 µM corresponding to 130 ng/µl, 390 ng/µl and 860 ng/ul) to 1 x 10^5 conidia ml^{-1} in liquid YEPD culture. Total proteins were then extracted from the fungus at 24 h, 48 h and 72 h after RNA inoculation. The fungal mycelia which grew in the liquid culture was isolated and ground up with liquid nitrogen. Ice-cold 50 mM Tris-HCl (pH 8) and 1 mM PMSF was added to the ground up mycelia. The samples were centrifuged at 4°C, at 10 000 rpm for 10 minutes. The supernatant was taken as the extracted protein sample. The concentration of each protein sample was determined using the Bio-Rad Protein assay (Bio-Rad, California, USA). GFP fluorescence was measured for 5 µg of each protein extract at final concentrations of 50 ng/µl in NUNC MaxiSorp™ dark 96 well plates (ThermoFisher Scientific) on a SpectraMax® Paradigm® (Molecular Devices, California, USA) at 488 nm excitation wavelength and 535 nm emission wavelength.

*C. zein*a conidia concentration of 1 x 10^5 spores ml^{-1} (2 ml) was added and spread onto cellophane disks (Bio-Rad) on corn meal agar (9 g CMA in 500 ml H_2O) (Merck, New Jersey, USA) in plastic Petri dishes (9 cm diameter). The fungus was left to grow for 1 week on the medium, where after dsRNA (800 ng) was inoculated onto the fungus directly using the Cz3_dsRNA construct for 48 hours before RNA extraction.

For both experiments including the fluorescent dsRNA and non-fluorescent dsRNA inoculation, three biological repeats were used. For the non-fluorescent dsRNA protein assay, there were four different individual treatments at 3 different time-points each. The statistical analysis used to process these results was a Two-way ANOVA analysis of variance ($p<0.0001$), followed by a Tukey's Multiple Comparison test ($p_{tukey} < 0.05$), using the GraphPad Prism v. 8.0 (GraphPad Software Inc., California, USA) software.

2.4 RNA extraction, RT-PCR and Sequencing

cDNA was synthesised from the Cz3_dsRNA product in order to sequence and confirm the genes expressed by the RNA. The dsRNA was synthesized into cDNA using the Maxima H Minus First Strand cDNA Synthesis kit (ThermoFisher Scientific). For the first strand cDNA synthesis the CSD F and PSD R primers were used which were located at the start and end of the dsRNA sequence. The cDNA was then used as the template in RT-PCR reactions using the three gene-specific primer pairs. The RT-PCR products were sequenced using DNA Sanger sequencing, using each specific gene forward primer (CSD F, PSD F and ECP F). The sequenced products were analysed and aligned using CLC Main Workbench 8.0.1 software (QIAGEN, Aarhus, Denmark).

Total RNA was extracted from *C. zeina* fungus growing on cellophane disks, which were placed on corn meal agar, and treated with Cz3_dsRNA, using QIAzol Lysis Reagent (Qiagen), as per the manufacturer's specifications. DNase treatment and RNA purification were done using TURBO™ DNase (ThermoFisher Scientific) and NucleoSpin RNA (Macherey-Nagel, Duren, Germany). RNA concentration and purity were analysed on a NanoDrop 1000™. The Maxima H Minus First Strand cDNA Synthesis kit (ThermoFisher Scientific) was used to synthesise cDNA, following the manufactures specifications and using 1 µg of total RNA and random hexamer primers. cDNA products were analysed using a RT-PCR reaction.

2.5 Real-time Quantitative PCR (RT-qPCR)

The expression of the specific target genes (*CHSD*, *PSD3*, *ECP2* and *GFP*) was analysed after the treatment with the Cz3_dsRNA using qPCR analysis. The RT-qPCR assay was performed using the SsoAdvanced Universal SYBR Green Supermix (BioRad, California, USA) following the manufacturer's instructions. The RT-qPCR reaction was set up using four biological replicates and three technical replicates. Quantification of gene expression was carried out using the BioRad CFX Connect Real-Time System (Bio-Rad). The gene expression data was normalized using the two reference genes, *GAPDH* and *40S* which were identified within previous studies (Fernandez et al., 2012) (Table 2.2, 2.3 and Figure 2.13-2.15). The data obtained from the RT-qPCR was analysed using an unpaired t-test statistical analysis between each respective gene and the water control, $p<0.05$.

2.6 MTT assay (Tetrazolium dye assay)

To assess fungal viability after treatment with Cz3_dsRNA, an MTT assay was used. *C. zeina* conidia (100 µl of $3x10^4$ conidia ml^{-1}) was added to a clear bottom 96 well plate. Cz3_dsRNA was added at a final concentration of 2 µg to the spores for 48 hours at room temperature. Two foliar fungicides,

Nativo (Bayer Crop Sciences, Leverkusen, Germany) and Artea (Syngenta, Basel, Switzerland) were used as positive controls, with water and GFP_dsRNA used as negative controls. The MTT Cell Viability & Proliferation Assay from ScienCell Research Laboratories (Carlsbad, California, USA) was used. At 48 hours after RNA inoculation the MTT reagent was added to each well at a 1/10 volume of the culture volume. This was incubated for 4 hours at 37°C, after which solubilization buffer was added for another hour before the absorbance was measured at 550 nm. The data obtained from the MTT assay was subjected to a One-way ANOVA analysis of variance ($p<0.0001$), which was followed by the Dunnett's multiple comparison test.

3. Results

3.1 dsRNA uptake by Cercospora zeina

To assess the capability of *Cercospora zeina* to take up externally applied dsRNA, three different *C. zeina* fungal cell structures, namely conidia, mycelia and protoplasts were treated with fluorescently labelled dsRNA for a period of 14 hours. Micrococcal nuclease (Mnase) treatment was applied to remove any excess unbound fluorescent RNA. Water (H_2O) and Fluorescein-12-UTP were used as negative controls and showed no significant fluorescence in the fungal cells' forms, before or after Mnase treatment (Figure S2.1). Confocal imaging was used to assess the RNA uptake by *C. zeina* cells. Wild-type *C. zeina* conidia treated with the fluorescent dsRNA showed selective RNA uptake within the cells. The difference between the RNA uptake in the conidial and mycelia cells before and after Mnase treatment indicated that after the excess fluorescent RNA is degraded, there still was a slight uptake of RNA within the cells (Figure S2.2 and S2.3). *C. zeina* protoplasts showed significant fluorescent dsRNA uptake after Mnase treatment (Figure 2.1). RNA was shown to primarily enter the edges of the protoplasts (Figure 2.1), similar to what was observed previously in *Botrytis cinerea* cells (Wang et al., 2016).

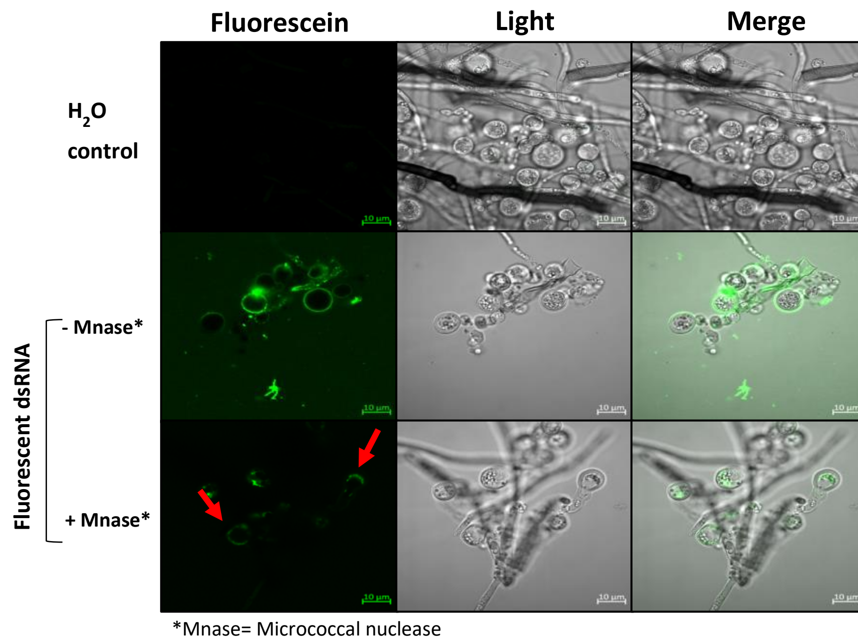

Figure 2.1: Confocal fluorescence microscopy showing dsRNA uptake by wild type *C. zeina* protoplasts at 10x magnification. LSM 880 confocal microscopy was used to observe fluorescence. Fluorescein-12-UTP wavelength at 495/525 nm was used. Water was used as negative control, and thus no fluorescence was observed in the water-treated samples. After Mnase treatment RNA continued to enter the protoplast cells, indicated by the red arrows.

3.2 GFP expression knockdown after treatment with dsRNA

Transgenic *C. zeina* which expressed GFP, *CzmCTB7* (Swart et al., 2017), was treated with dsRNA which targets a 325 bp region within the GFP gene, called GFP_dsRNA (Figure S2.4). The GFP fluorescence was observed using confocal microscopy at two different magnifications, 10x and 100x. When comparing fluorescence intensities at both magnifications (Figure 2.2), it is evident that after treatment with the dsRNA, less intense fluorescence can be observed, and that GFP knockdown took place.

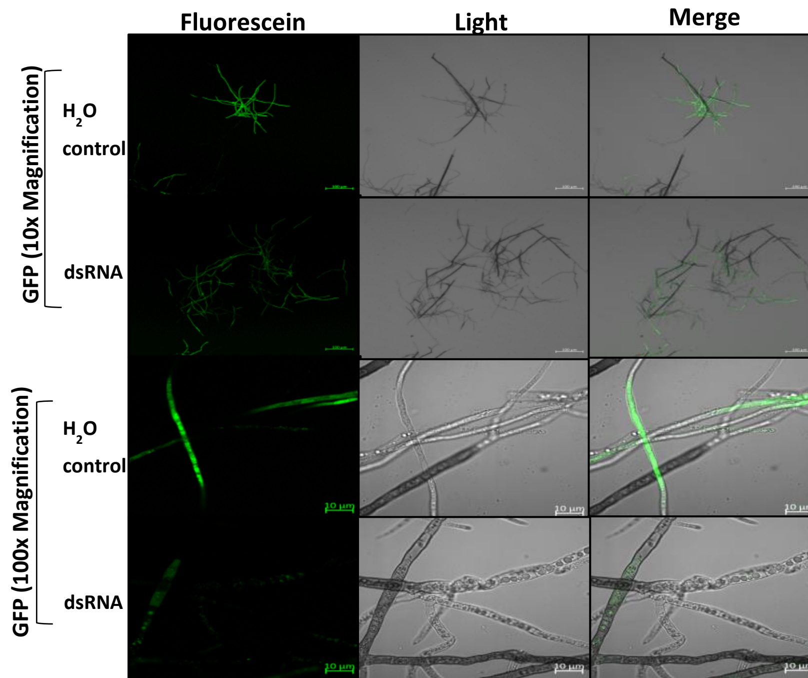

Figure 2.2: Confocal fluorescence microscopy indicating GFP knockdown using transgenic *C. zeina* mycelia. LSM 880 confocal microscopy was used to observe fluorescence. GFP fluorescent wavelengths of 488/509 nm was used. RNA treated transgenic mycelia samples showed less GFP fluorescence when compared to the water-treated control transgenic samples in both 10x and 100x magnification.

To further assess the capabilities of external dsRNA treatment on gene knockdown, the GFP_dsRNA was inoculated onto *C. zeina* mycelia, growing in liquid culture, at different concentrations and time points. Total proteins were extracted from the fungal cultures and the GFP protein fluorometric units were measured to assess the difference in knockdown caused by the external treatment of the fungus. The 0.3 µM RNA treatment did not have a significant decrease in Relative fluorescent units (RFU) at any of the time points compared to the water-treated samples, meaning that a higher concentration of RNA is probably necessary to induce gene knock-down. The 0.9 µM RNA concentration used had a significant decreasing effect on the of RFUs after 24, 48 and 72 hours respectively, making this a good concentration of RNA to use for silencing in the future (Figure 2.3). Concentration and time showed interaction, but only concentration had an effect on variance (The Two-way ANOVA table is indicated in Table S2.3). Surprisingly, when the high RNA concentration was inoculated onto the fungus for 48 hours and longer, the GFP gene regained function.

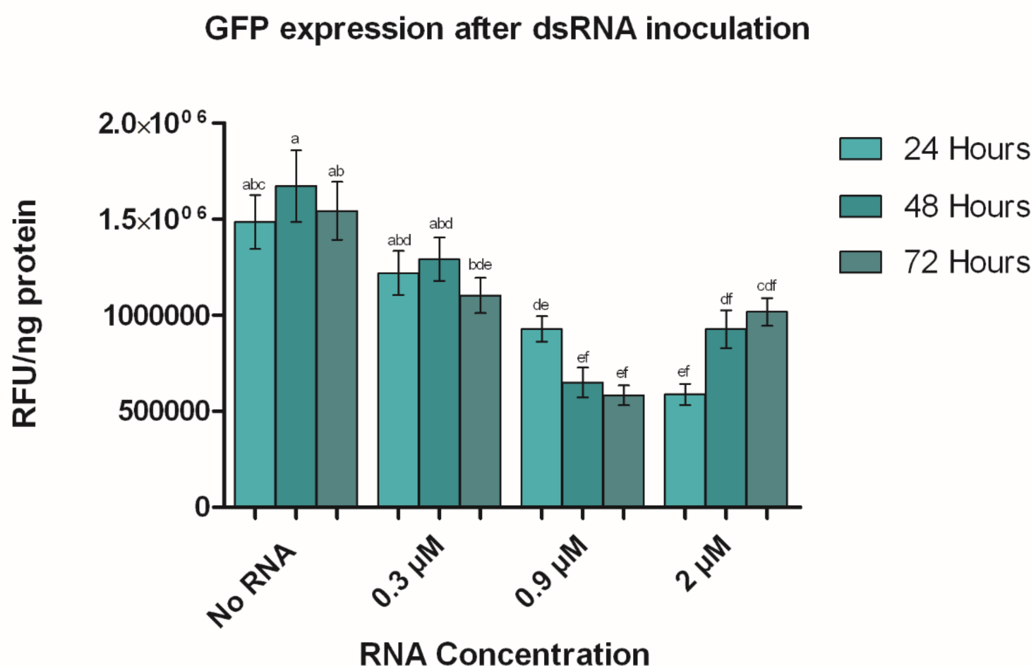

Figure 2.3: The relative fluorescent units (RFU) of GFP protein activity after treatment with different concentrations of GFP_dsRNA at different time points. The average RFU of each of the transformed *C. zeina* treated with dsRNA and evaluated at different time points. The 0.3 µM dsRNA concentration was not as successful in decreasing the GFP fluorescence when compared to the 0.9 µM and 2 µM concentrations. The 2 µM dsRNA concentration added for 24 hours showed the highest decrease in GFP fluorescence. Statistical analysis of the data was done using the Two-way ANOVA ($p<0.0001$) analysis of variance, followed by a Tukey's Multiple Comparison test ($p_{tukey} < 0.05$). (Error bars represent ± SEM, n=3). Means with different letters are statistically different.

3.3 C. zeina *gene-specific knockdown after treatment with dsRNA*

A double-stranded RNA construct which targeted regions within three *C. zeina* genes namely *ChsD* (798 bp), *Psd3* (404 bp) and *Ecp2* (201 bp) was created (Table S2.5 and S2.6 and Figure S2.5, S2.6 and S2.7) and this 1.4 kb product was cloned into a pJET1.2/blunt plasmid (Figure S2.7, S2.8 and S2.9). This dsRNA construct was called Cz3_dsRNA (Figure S2.5). When this fragment was analysed on a 1 % agarose gel using a DNA molecular weight marker, only a 750 bp product could be seen (Figure S2.10), whereas the 1.4 kb product was expected. The dsRNA product was synthesised into cDNA and sequenced using gene-specific primers to determine that the correct sequences were indeed targeted by the dsRNA. The sequencing results showed that the whole region of the *Psd3* and *Ecp2* genes was present, but only about a 350 bp sequence of the *ChsD* gene was present (Figure S2.11). This shortened dsRNA product was still sufficient to use, as after the dsRNA undergoes processing, small RNA fragments would still target different *ChsD* regions for gene knock-down. *C. zeina* growing on corn

meal agar was then inoculated with either the GFP_dsRNA or the Cz3_dsRNA and after 48 hours the total RNA was extracted. RT-qPCR analysis was conducted on each specific gene, using GAPDH and 40S as reference genes (Figure S2.13, S2.14 and S2.15 and Tables S2.2 and S2.3).

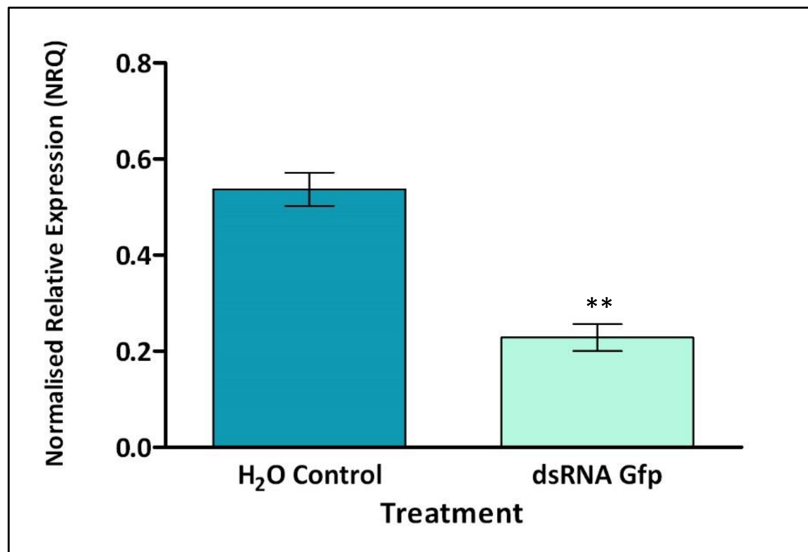

Figure 2.4: Normalized relative expression of *Gfp* within in vitro GFP-*C. zeina* after dsRNA targeting the GFP was added for 48 hours. The total RNA was extracted from the *C. zeina in vitro* samples growing on corn meal agar, 48 hours after treatment with GFP_dsRNA and the relative expression value of *Gfp* was calculated using RT-qPCR analysis. The relative expression values were normalized against the stably expressed reference genes, *GAPDH* and *40S*. RT-qPCR analysis showed that the expression of *Gfp* decreased significantly after dsRNA inoculation, when compared to the water-treated control. Error bars represent the standard error of the mean (SEM) (Unpaired t-test, $p<0.05$ [**] when compared to the control, n=4).

The expression of *Gfp* was significantly reduced when compared to the water-treated control (Figure 2.4). Around a 50% decrease in *Gfp* expression could be observed, signifying that this method of gene knock-down is effective. This *Gfp* RT-qPCR experiment was not linked to the previous results shown in Figure 2.3 but was done separately. In both experiments however, we saw a knock-down in *Gfp* expression. When looking at the specific *C. zeina* gene expressions, there was a difference in each gene's expression after treatment with the 3Cz_dsRNA construct (Figure 2.5). Only the *ChsD* gene showed a significant decrease in relative expression after dsRNA treatment. A similar decrease pattern as with the *Gfp* gene was observed where around 50% of the expression was lower compared to water-treated controls. Overall *Psd3* had the highest gene expression with *Ecp2* having the lowest gene expression (Figure 2.5). RT-PCR analysis of genes after cDNA synthesis (Figure S2.12), showed that the *Gfp* gene amplified a darker band, with the *ChsD* showing the second brightest band and the *Ecp2* the faintest band. This correlates to the results obtained after quantifying gene expression using RT-qPCR analysis (Figure 2.4 and 2.5). This difference in the decrease of the gene expression between different target genes was also observed in *Fusarium graminearum*, where a construct targeting three

different ergosterol genes was applied to the fungus, infecting it's host plant, and assessing each genes' expression shown to be differentially decreased (Koch et al., 2016).

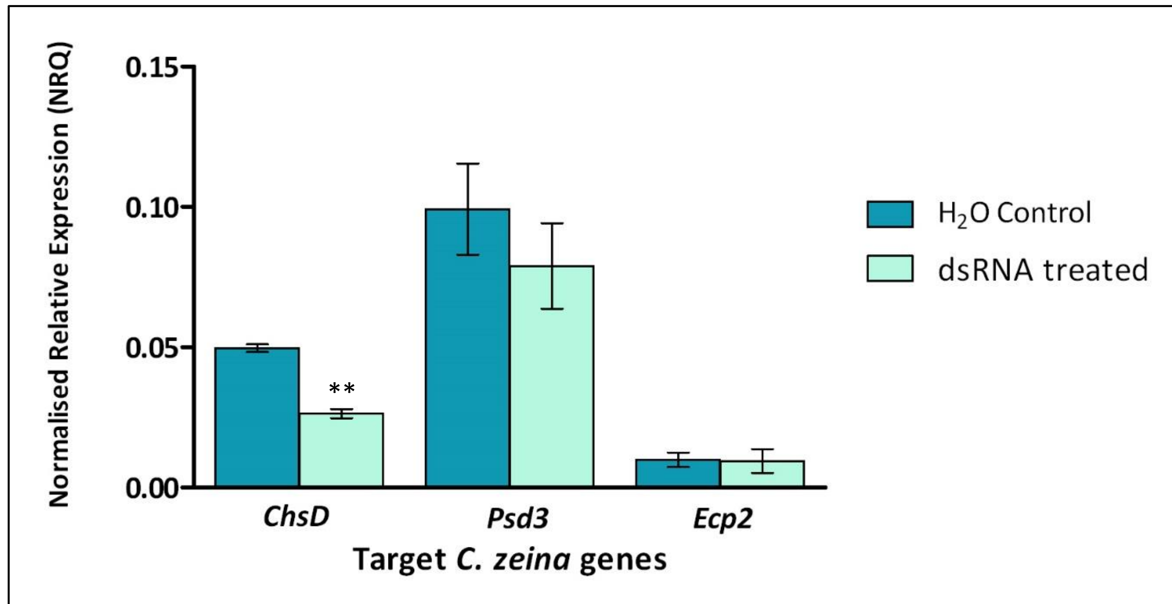

Figure 2.5: Normalized relative expression of target *C. zeina* genes after 48 hours of inoculation with the dsRNA construct *in vitro*. The total RNA was extracted from the *C. zeina in vitro* samples grown on corn meal agar 48 hours after treatment with dsRNA and the relative expression value of each target gene was calculated using RT-qPCR analysis. The relative expression values were normalized against the stably expressed reference genes, *GAPDH* and *40S*. The expression of *Psd3* was the highest in the fungus after the control water treatment. The *Ecp2* expression was the lowest in the fungus. The *ChsD* gene was the only gene that showed a significant decrease in expression after the addition of dsRNA, whereas *Psd3* and *Ecp2* showed no significant decrease. Error bars represent the standard error of the mean (SEM) (Unpaired t-test between each gene water control and RNA treatment, $p<0.05$ [**], n=4).

3.4 C. zeina *metabolic activity analysis after dsRNA treatment*

To assess whether the treatment of dsRNA onto the fungus, *C. zeina*, *in vitro* could have a decreasing effect on the fungal metabolic activity, an MTT assay was used. The MTT assay is a colorimetric assay in which the yellow tetrazolium salt, MTT, will be reduced to formazan crystals, changing from yellow to purple, in the presence of metabolically active cells, and could thus be used to assess the inhibitory effect of dsRNA on *C. zeina* cells *in vitro* (Van Meerloo et al., 2011). Fungal spores were treated with Cz3_dsRNA for 48 hours and the metabolic activity of the cells were analysed by adding the colorimetric substance MTT. Water and the GFP_dsRNA were used as controls. After Cz3_dsRNA was added onto the fungus, a 34 % decrease in the amount of fungal metabolic activity could be observed when compared to the water and GFP_dsRNA treated samples (Figure 2.6). Two fungicides namely Nativo (Bayer), a mixed mode of action fungicide, and Artea (Syngenta), a triazole fungicide were used as positive controls and in both cases, they showed a significant decrease of up to 55 % in

cell metabolic activity. Previously, Koch et al. (2018), assessed the potency of a dsRNA product directed against *F. graminearum* ergosterol genes and after using the MTT assay they reported that the cell metabolic activity decreased by 39 %.

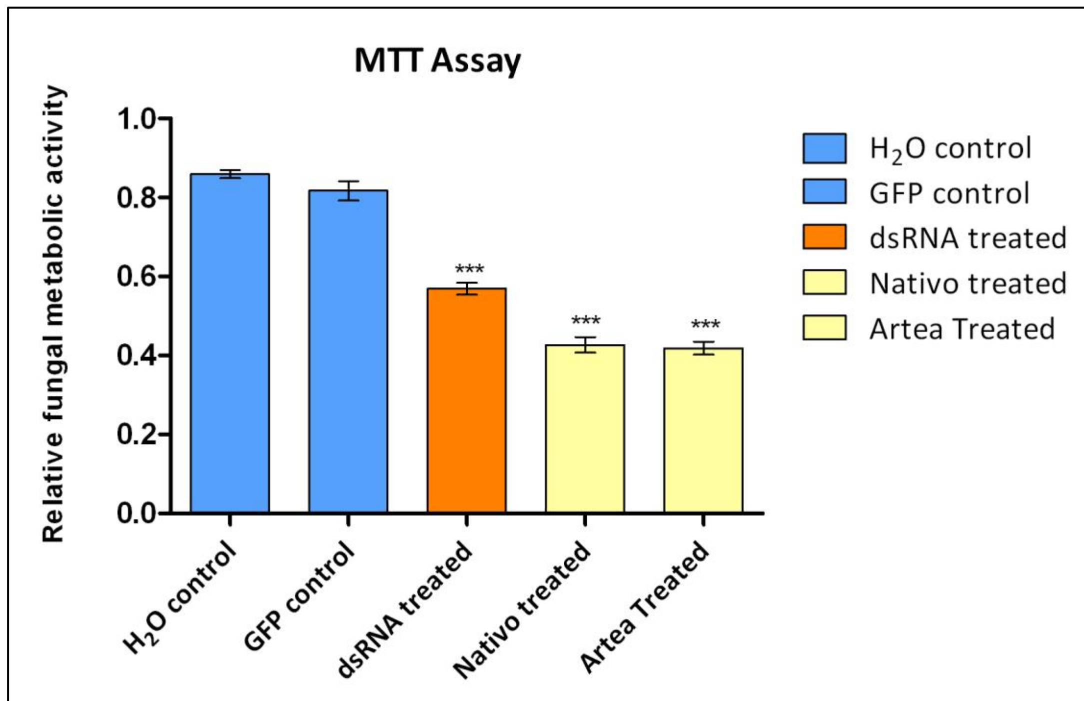

Figure 2.6: Relative *C. zeina* growth in vitro liquid culture measured by MTT viability assay after treatment with dsRNA and fungicide. $3x10^4$ conidia spores per 100 µl was incubated in YPD culture and treated with dsRNA and two different fungicides. MTT absorbance measurements were taken at 570 nm. Statistical analysis of the data was done using the One-way ANOVA analysis of variance ($p<0.0001$); followed by the Dunnett's Multiple Comparison Test comparing both H_2O control and GFP control the rest of the data. (Error bars represent ± SEM, n=3).

4. Discussion

The overall aim of this study was to evaluate if it is possible to use external dsRNA directed against the fungus *C. zeina* to silence specific gene expression. The main findings from this study were that (i) *C. zeina* is able to take up externally applied dsRNA; (ii) specific genes within *C. zeina* could be targeted for gene knock-down using externally applied dsRNA; and (iii) the *C. zeina* fungal metabolic activity could be downregulated using externally applied dsRNA.

C. zeina could successfully take up dsRNA, as shown in Figure 2.1 and 2.2. *Botrytis cinerea* has also previously been shown to take up dsRNA successfully within the spores and protoplasts (Wang et al., 2016). In this study the uptake of dsRNA by the fungal protoplast was shown to accumulate at the edges of the protoplast, possibly indicating that the dsRNA is not taken up by the vacuole entering the cytoplasm and nucleus. A similar trend was observed by Wang et al., (2016). The compartmentalized

uptake of dsRNA could be due to the composition of the vacuole, not allowing passage of the RNA. Specific degrading enzymes within the vacuole could also possibly degrade the dsRNA (Klionsky et al., 1990). Thus, when targeting specific genes using RNAi, the target gene location as well as the biology of RNA uptake needs to be kept in mind.

When creating the dsRNA, the DNA template was sequenced before using this in the transcription reaction (Figure S2.9). This indicated that the whole 1.4 kb fragment was used as the DNA template. However, after the transcription reaction, the dsRNA was analysed on a 1% agarose gel, and only a 750 bp product could be observed when comparing the product size to a DNA ladder (Figure S2.10). There are several possible reasons for observing a shorter dsRNA product on the gel including: (i) secondary structures could have formed in the final dsRNA product when it was run on the agarose gel (ii) dsRNA moves faster than dsDNA though the gel, and thus when comparing the RNA size to the DNA ladder it seems smaller (Livshits et al., 1990); (iii) During synthesis, the full length dsRNA product is not made, possibly due to secondary structures forming in the DNA template during synthesis. When sequencing this product formed by the dsRNA, a region within the *ChsD* gene was not amplified, indicating that the possible reason for this was the formation of secondary structures during synthesis. Although the full length of the *ChsD* gene region was not amplified within the dsRNA target, a sufficient part of this gene (± 350 bp) was indicated after sequencing, still rendering it sufficient to use as the dsRNA will get processed into smaller RNA pieces which target different regions within the *ChsD* mRNA (Figure S2.11).

Evaluating the silencing capability of the dsRNA targeting GFP in this study, the best gene silencing was observed at a concentration of 0.9 µM RNA (corresponding to 0.39 µg/µl), applied over a period of 48-72 hours. The higher concentration of 2 µM (corresponding to 0.86 µg/µl) dsRNA initially showed good silencing, but as time progressed, the silencing capabilities at this high concentration was lost. Targeting a *S. sclerotiorum* ethylene inducing peptide with dsRNA in a previous study showed a varied dose response to the RNA, with the lower concentrations (200 ng/ml) showing better silencing capacity than higher doses (McLoughlin et al., 2018). The doses used in this study was higher than the doses used by McLoughlin et al., (2018) as a larger area of fungal biomass was targeted, but the comparison between the dose and response could be observed as similar. The dose response for different genes targeted by previous studies varied, showing a saturation effect when using increasing doses of RNA (Asokan et al., 2014; Mitter et al., 2017). It is thought that at higher dosages the RNAi silencing machinery could be saturated with all of the RNA molecules to process, and is unable to process that amount of molecules at once (McLoughlin et al., 2018). Dubrovina et al. (2019), analyzed the silencing capacity of dsRNA directed against *NptII* and *eGfp* genes expressed by a transgenic *Arabidopsis thaliana. In planta* analysis of the silencing capacity of the dsRNA at different concentrations, showed that the 0.35 µg/µl had a better silencing effect than the higher concentration of 1 µg/µl (Dubrovina et

al., 2019), and this was similar to what we found. The optimal amount of dsRNA application for sufficient silencing depends on the type of target gene as well as the type of host plant on which it will be applied.

In this study a difference was observed in the degree of silencing between different target genes, where the dsRNA construct used targeted three different *C. zeina* genes, but only the *CshD* target gene showed a significant decrease in expression. The dsRNA doses and time-points used for the *C. zeina* specific genes were chosen based on the outcome of the GFP knockdown experiment. In 2018, McLoughlin et al., targeted specific *S. sclerotiorum* genes with a set concentration (500 ng/ml) of dsRNA, at different time points. They reported differences in the amount of decrease in gene expression between different target genes at different time points. The largest decrease in gene expression was observed 48 hours after inoculation with RNA targeting the gene encoding for thioredoxin reductase (McLoughlin et al., 2018). A different gene target, a mitochondrial translocase gene, showed a larger decrease in gene expression at 72 hours after RNA inoculation, when compared to the other time points. The maximum amount of decrease in gene expression for the thioredoxin reductase was 80 % where the decrease in expression for the mitochondrial translocase gene was only 60 % (McLoughlin et al., 2018). This could indicate that different gene targets could differ in optimal silencing time and efficiency of the silencing, which was also observed within this study and was one of the limitations of the results.

When comparing the RT-qPCR expression results with the RNAseq data obtained by Swart et al. (2017) there is a direct contrast. According to the RNAseq data on the fungus grown on corn meal agar, *Ecp2* was expressed the highest of the three target genes (10451 counts) and *Psd3* was expressed at the lowest (1852 counts) (Table S2.5), which is the opposite of what we saw in the RT-qPCR results, where *Psd3* was expressed at the highest level (Figure 2.5). This could have been because the fungus was only growing on the corn meal agar for a week, where it was sub-cultured from V8 media on which it grew for several weeks before that. When assessing the RNAseq data for the fungus growing on V8 media only (Table S2.5), the *Ecp2* gene was expressed at very low levels, similar to what we saw in our RT-qPCR results.

In this study different *C. zeina* gene targets showed a difference in gene expression knockdown, with only the *CshD* gene showing a significant reduction in expression. A variety of different fungal species and their respective target genes were targeted throughout the years, and in each case a difference in the amount of expression or disease decrease was observed (Table 1.3 in Chapter 1). Different *S. sclerotiorum* genes were targeted in a previous study, where the thioredoxin reductase showed a up to an 80 % decrease in expression, compared to another target gene, mitochondrial inner membrane translocase, only showing an expression reduction of 55 % (McLoughlin et al., 2018). The difference in gene knockdown between different targets could be due to a variety of factors, including the level of transcript, the mRNA half-life, the target gene secondary structure and accessibility, as well as the GC

content (Chan et al., 2009; Larsson et al., 2010; Shao et al., 2007). Higher GC content within RNAi targets have been shown to cause a decrease in the amount of RNAi activity, possibly due to the stronger triple bond between guanine and cytosine which causes a larger probability of stable secondary structure formation (Chan et al., 2009). Within this study the GC content for each gene was between 52-55 %, thus the probability that this caused the difference in gene knockdown is low. The level of transcript could have played a role with *Ecp2* being expressed very low, making silencing efficiency low, whereas *ChsD* was expressed at moderate levels and showed good expression knockdown (Figure 2.5).

When comparing the gene expression data with the MTT assay data, the gene silencing was not directly correlated with a loss in cell viability, where the gene expression decrease observed was higher than the cell metabolic activity decrease. It must be kept in mind that these two experiments were executed on different forms of the fungus. The MTT assay was conducted using liquid culture (YEPD media) and the expression analysis was conducted on solid media (V8 agar media), and thus these different growing environments could have affected how the fungus takes up the dsRNA. The expression of *ChsD* was decreased by up to 50 %, whereas the fungal growth was inhibited by only 34 %. This is similar to results observed by Koch et al., (2018) where they saw a strong decrease in gene silencing, but only an average decrease in the cell growth after dsRNA treatment. If the RNA construct contained other fungal metabolic target genes such as ergosterol, the fungal growth could have been decreased to a higher level, making consideration of what function each gene plays in the fungus important to note when choosing target genes.

The *ChsD* gene is important for synthesizing chitin which is an essential molecule which forms part of the carbohydrate skeleton of the fungal cell wall (Lenardon et al., 2010). Chitin is important for fungal stability and growth and that is why *ChsD* is a relatively good gene to target using RNAi. The production of chitin is associated with the biomass concentration of the fungus, thus factors which affect the quantity of chitin in the cell wall could influence the metabolic activity of the fungus, ultimately decreasing the fungal growth (Elsoud and El Kady, 2019).

In this study, a single strain of *C. zeina,* the Mkushi strain from Zambia, was used. Different results might be obtained when using a range of different isolates. *Cercospora zeina* within South-Africa has been shown to have high genetic diversity, and thus some strains might be more pathogenic than others (Muller et al., 2016). Nsibo et al., (2021) found that the Zambian population was distinct from other African populations and thus these fungal isolates may exhibit a difference in pathogenicity than isolates from other African regions. When deciding on using a RNAi strategy against a specific fungus, it is important to note the sequence diversity between different isolates and if there might be a difference in the uptake of the dsRNA between the isolates. Differences observed between fungal isolates might influence the success of implementing the RNAi strategy on a commercial level.

The research which could follow up from this study is an *in planta* analysis of the success of using external dsRNA against *C. zeina* infecting maize plants. A variety of practical factors needs to be considered before the *in planta* assay could begin, such as: (i) optimal environmental conditions for successful infection of *C. zeina;* (ii) maize hybrids used should be susceptible to GLS; (iii) method of inoculating *C. zeina* onto the maize plants; and (iv) timing, method and concentration of dsRNA application.

This study lays the groundwork for the development of an RNAi-based fungicide which targets cereal fungal pathogens, in this case specifically *C. zeina* which causes the maize yield limiting disease, grey leaf spot. From this study it is evident that the uptake of RNA by the pathogen target is important before external RNA can be studied. The choice of gene targets is very important, and complementarity within plant hosts needs to be considered. Timing and amount of RNA application is also important. The next step for this research will be to assess if this system is successful within the infected plant host and also considering nanotechnologies to stabilize the RNA. When all factors are considered, it could open up the possibility of developing an RNAi bio-fungicide against GLS.

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Chapter 3

Conclusion and prospects of using external RNA interference against crop diseases

This chapter was not written in the format for a manuscript and was written by me, Ingrid. Suggestions and comments were made by the two supervisors of this study.

As the world population is increasing, the food demand is escalating. Unfortunately, a variety of factors affect food security negatively including: climate change, pests and diseases. Plant pathogens are responsible for 10-16 % of global harvest losses, where in severe cases a global yield decrease of 40 % has been reported on maize, a staple food crop in Africa (Savary et al., 2012, 2019). One of the major yield limiting diseases on maize is grey leaf spot disease (GLS), which causes large necrotic lesions on the leaf blade leading to a loss in photosynthetic capacity of the plant (Ward et al., 1996, 1999). Currently one of the management practices against GLS is fungicide applications, which is unsustainable when overused due to resistance development of the fungus. This opens the field for developing novel strategies against devastating fungal diseases such as GLS.

New improved technologies could potentially help resolve the hunger problem. Researching new methods of pest and disease control is essential for agricultural progress and ultimately food security. The research and findings in this study is important as it is a step towards reaching one of the United Nations' Sustainable Development Goals, which is goal 2, defined as Zero Hunger. External RNAi has been shown to reduce the damage caused by pests and diseases by up to 50 %, therefore external RNAi-based fungicides and pesticides have the potential to increase global crop yields.

RNAi is a novel way of targeting certain protein coding genes for degradation and could be applied in the field of medicine and agriculture. The potential of using RNAi as therapeutic treatment in humans especially against harmful fungal diseases such as Aspergillosis has been deemed safer than conventional therapeutic agents, especially due to the potent effect that low concentrations of RNA has (Jain and Wadhwa, 2018; Ruddon, 2007). The RNAi mechanism has shown good potential as an agricultural control protecting crops from target pests such as the Colorado potato beetle, or diseases such as Fusarium head blight (He et al., 2020; Koch et al., 2016, 2018)

Host-induced gene silencing (HIGS) is also an RNAi-based effective strategy to combat fungal diseases, where you create a transgenic plant expressing a hairpin RNA targeting specific fungal genes for silencing. Using external dsRNA for fungal disease control, also called SIGS, has a few more advantages over using HIGS (Wang and Jin, 2017). HIGS require an established transformation protocol within the host and will thus be commercialized as a transgenic, whereas SIGS is a non-transgenic approach. With SIGS a variety of hosts can be sprayed once to target a variety of pathogens, whereas with HIGS, only the specific transgenic host and specific pathogen target will be protected. HIGS is limited to pre-harvest fungal protection, whereas SIGS can be applied post-harvest on the seeds or fruits for additional protection against disease.

External RNAi or SIGS could be advantageous over conventional chemical pesticides and fungicides as it is more environmentally friendly, more target specific and will not cause a build-up of resistance. Conventional fungicides such as strobilurins target a specific gene target within the fungus (present in most fungi) and if one nucleotide mutation occurs, the fungus will become resistant against the

fungicide (Fernández-Ortuño et al., 2008; Gisi et al., 2000, 2002). Using an RNAi-based fungicide, the targeting RNA will be separated into 20-22 nt pieces and each piece will individually target a region within the mRNA, making this RNAi fungal targeting method less sensitive to point mutations as well as more gene specific.

Targeting specific harmful organisms for degradation/destruction through RNAi requires careful consideration from the type of organism to the specific target genes within the organism. The organism chosen needs to be compatible with RNAi and the type of interaction that the organism has with its host needs to be considered. Target genes should preferably only be present within the harmful organism and not have any orthologs within the host. When considering genes to target, their level of expression should be intermediate, not too low or too high. Table 3.1 gives a summary of specific fungal species, their plant hosts and their target genes targeted either through HIGS or SIGS.

A few things to consider when targeting specific fungi using RNAi include: (i) if the fungus is able to take up externally applied dsRNA; (ii) if the fungal genome contains the correct RNAi silencing machinery genes; and (iii) if this type of interaction will work within the host-pathogen system. Specific fungi including *S. cerevisiae, U. maydis and C. gattii* are devoid of any RNAi pathway and RNAi machinery such as Dicer and Argonaute, possibly due to an evolutionary advantage or due to some organisms containing few transposable elements (Drinnenberg et al., 2011; Kämper et al., 2006; Laurie et al., 2008). Previously the wheat pathogen, *Zymoseptoria tritici,* was shown unable to take up external dsRNA or even process hairpin RNA through a HIGS system (Kettles et al., 2019). The phylogenetic analysis conducted in Chapter 1 on the Dothideomycete RNAi machinery showed that *Z. tritici* lacked an ortholog to Dicer-like 2, and this protein has been shown important in processing hairpin RNA. Certain physical or chemical barriers could also exist which hinder fungi from taking up external RNA and could be an important novel research topic.

Cercospora zeina, an important fungal pathogen affecting maize yields was chosen as the target organism in this study. The phylogenetic analysis revealed that *C. zeina* contains orthologs to all the important RNAi machinery genes. In this study it was shown that the fungus *C. zeina*, was able to take up externally applied double-stranded RNA (dsRNA). A transgenic *C. zeina* which expressed GFP was targeted by a dsRNA construct and the GFP fluorescence and expression was decreased by up to 50 %. A dsRNA construct targeting three *C. zeina* specific genes including chitin synthase (*ChsD*), phosphatidyl serine decarboxylase (*Psd3*) and the Effector protein 2 (*ECP2*) gene, showed a decrease in expression only for the *ChsD* gene. When more than one gene is targeted through RNAi, some genes' expression can be silenced more than others, in this case *ChsD*, possibly due to their level of expression or their level of accessibility for RNAi machinery. The fungal metabolic activity of the *C. zeina* fungus was also decreased by 40 % after uptake and processing of the exogenous dsRNA.

Table 3.1: List of pathogenic fungal genes targeted through HIGS/SIGS

Fungal target	Host	Gene target	Gene Function	Used in HIGS/SIGS	Reference
Blumeria graminis	Barley Wheat	*Avra10*	Virulence effector	HIGS	(Nowara et al., 2010)
Botrytis cinerea	Arabidopsis Tomato	*DCL1* *DCL2*	Plays a role in growth and pathogenicity	HIGS & SIGS	(Wang et al., 2016)
Fusarium culmorum	Wheat	*FcGlc1*	Important for cell wall biogenesis	HIGS	(Chen et al., 2016)
Fusarium graminearum	Barley	*AGO* *DCL*	RNAi silencing machinery Vegetative growth Disease development	SIGS	(Werner et al., 2020)
Fusarium graminearum	Banana	*Ftf1*	Fungal growth, development and pathogenesis	HIGS	(Ghag et al., 2014)
Fusarium graminearum	Barley	*CYP51*	Plays a role in Ergosterol biosynthesis	HIGS & SIGS	(Koch et al., 2016; Koch et al., 2013)
Fusarium graminearum	Wheat	*Chs3b*	Catalysis the biosynthesis of chitin	HIGS	(Cheng et al., 2015)
Fusarium oxysporum f. sp. *lycopersici*	Tomato	*FOW2* *ChsV*	Plays a role in fungal pathogenicity	HIGS	(Bharti et al., 2017)
Sclerotinia sclerotiorum	Tobacco	*Chs*	Catalysis the biosynthesis of chitin	HIGS	(Andrade et al., 2016)

Possible targets for fungal RNAi pathogenic genes should include genes that are essential for the fungal life cycle as well as genes important for pathogenicity. The Ascomycete, *Magnaporthe oryzae*, contain calcium signalling related genes which has been shown to play a role in fungal pathogenicity, hyphal growth, appressorium development and sporulation, making them promising RNAi targets (Nguyen et al., 2008). Cell wall degrading enzyme genes such as endoxylanases and cellulases were shown to be important for *M. oryzae* virulence could also be possible RNAi targets (Van Vu et al., 2012). Effector genes which has been shown to play and important role in the virulence of the fungus could also be effective RNAi targets and could ultimately lead to a gain in resistance by the host plant (Bhadauria et al., 2015; Schaefer et al., 2020). Another gene family which could be targeted is the genes important for nitrogen allocation to pathogenic fungi, including the Allantoicase and Urease genes (Benatto Perino et al., 2020). These genes were showed to be important in helping the fungus to establish disease and could potentially protect the host plant when targeted through RNAi (Benatto Perino et al., 2020).

Polyamines (PA) are essential for the fungal metabolism, affecting fungal growth and development, and through the targeting of PA biosynthesis genes using RNAi, mycelial growth decreased significantly within the fungus *Aspergillus nidulans* (Khatri and Rajam, 2007). Targeting the *F. graminearum* cytochrome P450 lanosterol C-14αdemethylase (CYP51) gene family, which is essential for ergosterol synthesis, also showed decreased fungal growth and development. The formation of fusarium head blight disease was decreased significantly after a HIGS strategy targeted the chitin synthase gene which catalyses the formation of chitin, essential to the fungal cell wall (Cheng et al., 2015). All of these above-mentioned genes could be good targets for RNAi against *C. zeina* and should be looked at together with the specific RNA-sequencing data to establish which genes show relative high expression before infection.

Dicer-like (DCL) and Argonaute (AGO) proteins within the *B. cinerea* and *F. graminuearum* fungi have been shown to play an important role in pathogenicity and growth (Gaffar et al., 2019; Weiberg et al., 2013; Yu et al., 2018). It was thought that these genes are essential for processing the exogenous dsRNA and establishing the RNAi process, however when these genes were targeted through RNAi, using SIGS, a decrease in fungal growth within the host plant was observed (Wang et al., 2016; Werner et al., 2020). It was previously though that the fungal silencing machinery controlled the molecular mechanisms of SIGS, but after the successful silencing of these fungal RNAi genes using SIGS, it is speculated that the plant silencing machinery is involved (Werner et al., 2020). The SIGS phenomenon probably works in a cross-kingdom way where the plant's RNAi components such as Dicer and Argonaute process the incoming dsRNA into sRNA and this is then targeted towards the respective fungal genes which are infecting the plant. The possibility of targeting fungal DCL and AGO proteins, through SIGS, have been shown promising for promoting plant health and decreasing disease incidence (Wang et al., 2016; Werner et al., 2020). There are a lot of unknowns regarding the mechanism of SIGS

and how the dsRNA is processed, and by understanding these processes, this mechanism can be optimised and utilised to its full potential.

Targeting multiple genes and pathogens at once could also have a more potent effect than when applying one gene target at a time and could also make the RNAi fungicide more broad-spectrum (Rodrigues et al., 2018). Previously, both the *B. cinerea* as well as *Verticillium dahlia* infections were decreased when a transgenic *Arabidopsis* was created which expressed a hairpin RNA construct targeting both *V. dahlia* and *B. cinerea* Dicer-like1/2 genes simultaneously (Wang et al., 2016). When two or even three genes within a fungus are targeted through RNAi simultaneously, the fungal growth is inhibited more effectively, when compared to single targets (Koch et al., 2019). Looking for target gene homologs between different fungal pathogens such as *S. sclerotiorum* and *B. cinerea* could potentially cause a host plant to be protected against a range of pathogens using a single RNAi-based fungicide (McLoughlin et al., 2018). When deciding to create a broad-spectrum RNAi fungicide it is very important to assess the effect that the RNAi fungicide will have on beneficial endophytes within the environment.

The right time of application of fungicides are very important, depending on what host-pathogen system is targeted, and thus the application of RNAi bio-fungicides will also need to be optimised. It is important to assess if the RNAi bio-fungicide will work in a preventative or curative manner. This will depend on the fungal invasion strategy, for example *C. zeina* has a long latent period which could cause preventative spray ineffective if the RNA is not stable within the plant for a long period of time. *B. cinerea* on the other hand has a very short infection period after penetration, and externally applied dsRNA have been shown successful against disease when the dsRNA was administered two days before infection, thus preventatively (Wang et al., 2016). Aspects which needs to be considered when thinking about using RNAi bio-fungicides include: (i) if and how the RNA is able to enter the specific host plant; (ii) how long the dsRNA stays stable within the plant; (iii) if the fungal or plant RNAi machinery will be used to process the RNA; and (iv) if the RNA should be sprayed onto the plant before or after fungal infection (preventative/curative).

When assessing optimal fungicide spray conditions, it is important to take into account the type of host plant as well as the fungal pathogen life cycle. Bradley et al., (2020) showed that the optimum timing for applying a range of mixed fungicides to maize (*Zea mays*), to be most effective in disease control and yield preservation, is at the silking (R1) stage. Application of the fungicides pre-R1 stage showed a poorer level of GLS control compared to only applying the fungicide during R1 stage (Bradley et al., 2020; Ward et al., 1997). Thus, when applying RNAi bio-fungicides the same principles will count, and for effective GLS disease control, the bio-fungicide needs to be applied just after the last V stages, when the maize reaches the R1 stage.

Challenges associated with using SIGS as an RNA bio-fungicide control include: (i) how long the effect of the dsRNA will last after spray application. The dsRNA could be coated with nanoparticles which could help keep it stable for longer (Mitter et al., 2017); (ii) the mode of dsRNA uptake by plants are still unknown and how long the dsRNA persists within the plant is another unknown; (iii) the cost of using these SIGS could initially be more expensive than fungicides, but making large amounts of dsRNA has become increasingly cheaper; and (iv) the effect that these SIGS could have on other beneficial fungi needs to be thoroughly studied before commercialization.

Layered double hydroxide (LDH) clay nanoparticles which is a type of inorganic layered material are non-toxic and non-degradable and could be used to stabilize dsRNA (Mitter et al., 2017). The nanoparticle is positively charged while the RNA is negatively charged, allowing them to bind to each other. When the nanoparticle reacts with atmospheric CO_2 and humidity, it will form carbonic acid which will then gradually release the dsRNA (Mitter et al., 2017; Niehl et al., 2018). Such LDH clay nanoparticles have been used together with dsRNA targeting plant viruses and were shown to cause protection and be detectable on leaves, 30 days after the spray application (Mitter et al., 2017).

The implementation of nanotechnologies in agriculture is very promising on different scales including pest/disease management as well as nutrient utilization. However, certain barriers against the use of these technologies include efficient field scale delivery as well as safety concerns (Hofmann et al., 2020). One controversial aspect of using nanotechnologies for disease management in agriculture is that the nanomaterials is released directly into the environment and could potentially be introduced to food. Consumers will have to be educated and guaranteed their safety when consuming such products. These nano products, including food, cosmetics and biocides must be regulated thoroughly, but unfortunately at the moment in the European Union there is uncertainties regarding the regulation of nanomaterials (Miernicki et al., 2019).

RNAi bio-pesticides/bio-fungicides could be commercialized in a range of different forms including sprays, root drenching, stem injections, seed treatment and powder form, depending on what disease or pest is targeted (Cagliari et al., 2019). The RNAi biocontrol could also be incorporated during post-harvest storage and transport of grain, fruits and vegetables, protecting the products from fungal and pest damage (Majumdar et al., 2017).

There are different ways in which dsRNA could be administered onto plants. This depends on the type of plant as well as the type of pathogen target. Grapevines which needed protection against *B. cinerea* using dsRNA treatment, was treated with the dsRNA in three different ways, including spraying the leaves at high pressure, adsorbing the dsRNA onto the petioles and spraying bunches with the dsRNA postharvest (Nerva et al., 2020). The most effective dsRNA treatment against *B. cinerea*-infected grapevines was the high-pressure spray treatment on the grape bunches (Nerva et al., 2020). For the continuation of this study, it would be advantageous to test different ways in which the RNA could be

applied onto the maize leaves, whether it is through high pressure spray, a dripping/painting method or using a fine misting system.

In line with precision agriculture, unmanned aerial vehicle (UAV) aircrafts, commonly known as drones, could be helpful in applying such environmentally safe RNAi-based pesticides and fungicides to smaller field areas (Mogili and Deepak, 2018). The use of UAVs for aerial bio-pesticide/bio-fungicide application could be preferred above the normal traditional aircraft as they are able to fly lower and hover in one place for a certain period of time, and they are also smaller, increasing the precision and speed of the spray application (Linn, 2015; Qin et al., 2016). A barrier in using UAVs for spray application is the regulatory framework and extensive rules pertaining to the flying of these aircrafts, especially in the United States (Linn, 2015; Petty and Chang, 2018). With technology evolving rapidly, and hopefully policies evolving together with the technologies, the use of UAVs in agriculture could become the new norm.

Recently Monsanto and DowAgroSciences have collaborated on a seed variety product which is a combined trait product, targeting the corn rootworm (CRW) pest complex, which works like the HIGS principle (Head et al., 2017; Cagliari et al., 2019). This product is still being considered for registration under the US EPA and depending on approval, will be commercialized under the name SmartStaxPRO®, and contains the Cry3Bb1 gene, as well as a novel RNAi-based trait which targets a sucrose non-fermenting (*Snf*) gene of the western corn rootworm (WCR) (Head et al., 2017). Upon consumption, the maize produced dsRNA which targets a 240-bp fragment of the CRW *Snf7* gene, leads to a downregulation of the gene and ultimately insect mortality. This SmartStaxPro® variety was compared to other varieties also containing the *Bt* proteins but without the added RNAi traits, and it was shown that the root damage to the RNAi-containing varieties was much less compared to those without RNAi, indicating that stacked maize varieties targeting both Cry proteins and specific mRNA sequences are very effective in reducing insect damage (Head et al., 2017).

Bayer Crop Science owns the BioDirect™ Technology which forms part of their agricultural biological platform where they focus on using RNAi to develop better disease and pest resistance, increase yield or improve crop quality. The details of this technology have however not been publicly exposed, but it is believed that this technology will aim to develop a glyphosate-resistant weed control, control insects and viruses and improve the health of honeybees (Zotti et al., 2018). Syngenta has also begun to create RNA-based bio-controls targeting the Colorado Potato Beetle (Bramlett et al., 2019)

Conventional pathogen control, such as fungicides, are expensive and in South-Africa, farmers will pay between R250-R450 per hectare (AECI Plant health, Woodmead, Gauteng), making up about 21% of the total input costs. When looking at alternative control methods such as external RNAi, for large scale management of pests and diseases, an estimate of between 2 to 10 g of dsRNA per hectare is

needed, depending on the type of RNAi target (Zotti et al., 2018). Meeting such large-scale dsRNA demands will not be cost effective when using *in vitro* transcription methods, where some companies ask up to R30 000 for 1g of dsRNA (RNAGreentech, Texas, USA). Low cost large-scale manufacturing of dsRNA is thus required where certain companies use engineered bacteria and yeast to produce the dsRNA on large scale. Due to the growing demand for large quantities of these dsRNA to be used specifically in the agricultural sector, the cost has significantly reduced and could be up to R30 per 1g of dsRNA (Zotti et al., 2018). In the future, alternative crop protection methods could become more cheaper as the technology evolves and the demand increases.

For the continuation of this study it is important to analyze different *C. zeina* target genes for silencing both *in vitro* and *in planta*. The *C. zeina* RNA seq data (GEO dataset: GSE94442 and GSE90705) together with published data on other fungal gene targets (Table 3.1) could be used to identify good gene targets within the *C. zeina* genome. Knock-out mutants of certain *C. zeina* genes could be created to analyze the importance of the genes for the specific fungal growth and pathogenicity, and RNAi gene targets could be chosen from there. The criteria described earlier also needs to be considered when evaluating genes as possible targets. Tool-based approaches could also be used where a specifically designed program will predict the best targets for siRNA generation (Ahmed et al., 2020)[1]. dsRNA target constructs can be analyzed for their target specificity and their silencing efficiency using the software called SI-FI (Lück et al., 2019)[2].

The future research which can continue from this study includes analyzing the success of using RNAi against *C. zeina* during the infection of maize plants. The research aspects could include different ways in which dsRNA is applied, as well as different time points of application. Creating nanoparticles coated with dsRNA to allow the RNA to stay viable on the plant for longer is also a novel research topic which could be explored. The *in planta* challenges for the continuation of this study includes the following: (i) the successful uptake of dsRNA by the maize plant, and how this is facilitated; (ii) how long the specific dsRNA will be viable within the maize plant; (iii) whether the dsRNA will be processed by either the plant or the fungal RNAi machinery; and (iv) the optimal timing of the dsRNA application needs to be assessed as this fungal pathogen has a long latent period within the plant before any onset of symptoms.

The findings of this study lay the groundwork for future RNAi-based studies against maize yield limiting pathogenic fungi. RNAi-based pesticides and fungicides are part of the "New Green Revolution" where agriculture is adopting new technologies which are efficient and effective, minimizing the harmful effects on both humans and the environment (Helmstetter, 2020).

Footnotes

[1] http://plantgrn.noble.org/pssRNAit/

[2] http://labtools.ipk-gatersleben.de/

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Appendix A

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Botrytis         716 TKVHYSNNLSTKVVALLSILKDRFQRP-TNDKCIVFVKERYTARLLASLLSTPEA----G
Cercospora       405 QLTDVRSDVSSKVYELHRYLQLQFERT-SDHRCIVFVDRRYTARMLHNLFL--QI----S
Cercospora_1     421 QPTNVRSDVSSKVYELHRYLQLQFERT-SDHRCIVFVDRRYTARMLHNLFL--RI----S
Dissoconium      427 GTTTTTNIMSRKVARLLQYVTPEFARD-NEQRCIIFVDWKQTARLLCQLVT--EI----G
Fusarium         396 ---PQSQELSAKVKCLHEILVHAFTVD-NTKRCIVFVDQRHTACLLSDLYD--QVSM--A
Neurospora       440 ---LDPTMLSSKVIMLVRILRDQFERGVGAQRCIIFVRQRNTAMLLADLLQ--QPEIKSH
Sclerotinia      750 TKVHYSTNLSTKVVALLSILKDRFQRP-TNDKCIVFVRERYTARLLASLLSTPEA----G
Sphaerulina      422 ----VHADISSKVLELHRYLRLQFERL-SDHRCIVFVDRRYTARMLHILFS--RL----S
Zymoseptoria     431 A-TVTADDVSSKVWSLRGHLDAQFERP-SNQRCIVFVDQRYTARLLTRLFQ--KI----G
Zymoseptoria_1   420 A-TVTADDVSSKVWSLRGHLDAQFERP-SNHRCIVFVDQRYTARLLTRLFQ--KI----G

Botrytis         771 TPFLKAAPLVGTTSA-S--AGEMHITFRSQTLTMHNFRNGKINCLIATSVAEEGLDIPDC
Cercospora       458 TPHMRGHFLVGSNNG-G--LDEDSFSFKQQVLTLLKFRKGELNCLFATSVAEEGLDVPDC
Cercospora_1     474 TPHMRGHFLVGSNNG-G--LDEDSFSFKQQVLTLLKFRKGELNCLFATSVAEEGLDVPDC
Dissoconium      480 INNLRPGFVTGSGKDDK--LDRSTFSLRSQVLSLTHLRSGEVNCMFATSVAEEGLDIPSC
Fusarium         448 IPGMNASYMIGQQSS-S--STLGNMSLRKQCSTLKNFRDGVINCLFATSVAEEGIDIPSC
Neurospora       495 IPSIAAEVLVGGGTT-GSSYVNAKINFQQQNRIIRKFKLGEINCLFATSVAEEGLDIPDC
Sclerotinia      805 TPFLKVAPLVGTTST-S--AGEMHITFRSQTLTMHDFRNGKINCLIATSVAEEGLDIPDC
Sphaerulina      471 TPHMRGHFLVGSNNG-G--LDEDSFTFRQQVMTLMRFRKGEINCLFATSVAEEGLDVPDC
Zymoseptoria     483 TKHLHSQFLIGHGSG-N--ADEGSFTFRQQVWTLLKFRKGVFNCLFATSVAEEGLDVPDC
Zymoseptoria_1   472 TKHLHSQFLIGHGSG-N--ADEGSFTFRQQVWTLLKFRKGVFNCLFATSVAEEGLDVPDC
```

Figure S1.1: Alignment of different fungal DCL-1 proteins. Protein orthologs identified to the *Neurospora crassa* (Neurospora) DCL-1 protein were aligned to fungal species including, *Botrytis cinerea* (Botrytis), *Cercospora zeae-maydis* (Cercospora), *Cercospora zeina* (Cercospora_1), *Dissoconium aciculare* (Dissoconium), *Fusarium graminearum* (Fusarium), *Sclerotinia sclerotiorum* (Sclerotinia), *Sphaerulina musiva (Sphaerulina), Zymoseptoria ardabiliae* (Zymoseptoria) *and Zymoseptoria tritici* (Zymoseptoria_1). The figure shows a region within of the amino acids sequences aligned to each other. Although the sizes of each protein is different, there are still conserved regions within each fungal protein.

```
Botrytis      425 LGIIAQQ-KPPFSAIIFVQERATVSVLAHLLSHHPLTKDRFKIGTMVGTSLNGKRTDQIG
Cercospora    373 VATLQQHADATVRCIVFVEQRVQVTALAEMLRRVPELQNLYSVAAFVGTSTNTNRKVSLA
Cercospora_1  410 IATLKQHADATVRCIVFVEQRVQVTALAELLRRMPEMQNIYSVAAFVGTSTNTNRKVSLA
Dissoconium   360 VATLLSSASTSMRGIIFVEQRAVTTAMCHLLNNIPEIAAHYNIGSFVGTSANTSRKIRVA
Fusarium      433 IQELLAA-DEDVVGIIFVRSRAAANVLCALLREHPEIRQRYRVGSVVGSAATKIRKQNIY
Neurospora    271 LKVLASH-QQDPVGIVFVKERVMVSIVTHIISTHPLTKDRYRTASMIGTASVPGKARNHM
Sclerotinia   458 LNIILQQ-EPPFSAIIFVEERATVFVLADLLSQHPLTKERFKIGTMVGSSSNSKRTQIVG
Sphaerulina   378 LQILLQHAEPGVHCLIFVEQRVQVTALAELLRRVPALEDSYKIAGFVGTSTNTNRKISVA
Zymoseptoria  354 LDYLLQDPGDELCGIVFVERRVVASVLSSLLNNHPAANGRLRCIPSVGGSSFSGRKFAVT

Botrytis      484 ELVDVNQQKDTLSSFKRGKIDILIATNVLEEGIDVPACNLVICFSKPANLKSFVQRRGRA
Cercospora    433 DLVAIKDQEHDLQAFREGRKNLMIATNVLEEGIDISACNLVICFDAPKNLVSFVQRRGRA
Cercospora_1  470 DLVAIKDQEHDLLAFREGRKNLMIATNVLEEGIDISACNLVICFDAPKNLVSFVQRRGRA
Dissoconium   420 DLVELKQQQSSLGEFRDGSKNLIIATNVLEEGIDVSACNFVISYDPPQNLVSFVQRRGRA
Fusarium      492 EYLP-GATADTLRDFKTGAINLLVSTSVLEEGIDVAVCNLVICFDETTTLKSHIQRRGRA
Neurospora    330 DMTK-KEDMTSLEGFRLGRFNLLVATSVLEEGIDVPICNLVICFDEPSNIKSFIQRRGRA
Sclerotinia   517 ELVDLDKQKDTLSRFKLGKIDILIATNVLEEGIDVRACNLVICFSKPSNLKSFIQRRGRA
Sphaerulina   438 DLVALSDQSKDLQAFRDGHKNVMVATNVLEEGIDISACNLVICFDAPKNLVSFVQRRGRA
Zymoseptoria  414 ELVDWRAQRQALATFRRGDANIIVATSVLEEGIDVQACNVVACFDFPANLKSYIQRRGRA
```

Figure S1.2: Alignment of different fungal DCL-2 proteins. Protein orthologs identified to the *Neurospora crassa* (Neurospora) DCL-2 protein were aligned to fungal species including, *Botrytis cinerea* (Botrytis), *Cercospora zeae-maydis* (Cercospora), *Cercospora zeina* (Cercospora_1), *Dissoconium aciculare* (Dissoconium), *Fusarium graminearum* (Fusarium), *Sclerotinia sclerotiorum* (Sclerotinia), *Sphaerulina musiva* (Sphaerulina), *Zymoseptoria ardabiliae* (Zymoseptoria) *and Zymoseptoria tritici* (Zymoseptoria_1). The figure shows a region within the amino acids sequences aligned to each other. Although the sizes of each protein is different, there are still conserved regions within each fungal protein.

```
Bot      860 PKDTDSADS-------K-SNCPAGTVVDRGVTES----RNWDFFLQPHQSLMGTARPCHY
C.       821 PTRKEDADE-------RSWNPRPGTVVDRGIVGK----IVREFYLQAHQGLQGTARPAHY
Cerc     792 CADSRDSYTQ-GK-NGINGNAKPGLLVDTVITQPITKDGFRDFFLQSHAAIRGTAKSAHY
Cerc_1   880 PTRKEDADE-------RSMNPKPGTVVDRGIVGK----IVREFYLQAHQGLQGTARPAHY
Dis      848 PTTLAQTS--------RSSNPQPGTVVDRAITSK----ILFDFYLQAHDALQGTARPAHY
Fus      891 PTSSESMTS-------K-NNIENGTIVDRGVTQA----RYWDFFLTAHSSIKGTARPAHY
Neu      944 PTDPKHIHF-------KSKSPKEGTVVDRGVTNV----RYWDFFLQAHASLQGTARSAHY
Neu_1    930 PTDPKHIHF-------KSKSPKEGTVVDRGVTNV----RYWDFFLQAHASLQGTARSAHY
Scl      898 PKDAESADS-------K-SNCPAGTVVDRGVTES----RNWDFFLQPHQCLMGTARPCHY
Sph      884 PTRVEDADYN-PQRDKGSWNPLPGTIVDRGIVGK----VIREFYLQAHQGLQGTARPAHY
Sph_1    692 PTEPNNTK------LALKENCLPGTVVESDITSP----YYFDFFLQSHAVEKGSAKPTHY
Zymo     953 PIGPPT---------G-KKGNCVPGTVVDSHITSP----YYFDFYLQSHEVMEGSAKPTHY
Zymo_1   813 PLDDKDCVQK-FNGQRKNDNVKPGLVVDQVITHP----FSSDFYLQSHDPIIGTGKSAHY
Zymo_2  1002 PTRDADCVRKRPNFQRKSENVQPGLVVDQVITHP----YSSDFYLQSHDAIIGTGKSAHY
Zymo_3   887 PLSDKDEN------MTATWNCKPGMVVDTHITSP----LYMDFFLQSHDVEKGSAKPTHY

Bot      908 FVILDEIFRSQKVKAPHTTSADSLEELTHNMCHLFGRATKAVSLCPPAYYADLLCTRMRA
C.       870 VVIKDDIS----------FSADALEQFTHHLCYLFNRATKAVSICPPAYYADLLCDRGRS
Cerc     850 IVIRNDKKC---------NLDMSAIHNITHAFCYNYARATKGVSYCAPAYYADRLCDRMHG
Cerc_1   929 VVIKDDIS----------FSADALEQFTHHLCYLFNRATKAVSICPPAYYADLLCERGRC
Dis      896 VVVKDQIG----------FSANQLQTFTHQLCYLYNRATKAVSICPPAYYADLLAERGRC
Fus      939 TVLLDEVFRAKY----GAEAANELERYAHELCYLFGRATKAVSICPPAYYADVVCTRARC
Neu      993 TVIVDEIFRADY----GNKAADTLEQLTHDMCYLFGRATKAVSICPPAYYADLVCDRARI
Neu_1    979 TVIVDEIFRADY----GNKAADTLEQLTHDMCYLFGRATKAVSICPPAYYADLVCDRARI
Scl      946 FVILDEIFKSQKVKAPHQTSADSLEELTHNMCHLFGRATKAVSLCPPAYYADLLCTRMRA
Sph      939 VVIKDDIS----------FTADALEQFTHHLCYLFNRATKAVSICPPAYYADLLCERGRA
Sph_1    742 FVIENEMK----------FDQDSLQNVTNGLCYNISHSTGFVSYASPAYLADRLCERVSL
Zymo    1000 FVLENGMD----------FTEHELQNLTNNFCYVFSHSNSAVSYVAPAYFADKLCERAML
Zymo_1   868 FVLQNGMG----------LTTAQLQSTTHDFCYAYARATKGVSYCAPAYYADRLCDRARA
Zymo_2  1058 FVLQNGMA----------LTTAQLHSTTHDFCYAYARATKGVSYCAPAYYADRLCDRARA
Zymo_3   937 FVLENGMK----------LKEAELQDLTNSFCYTFQHSTSAVSYPAPVYAADKLCERLML
```

Figure S1.3: Alignment of different fungal QDE-2 proteins. Protein orthologs identified to the *Neurospora crassa* (two copies, Neu and Neu_1) QDE-2 protein were aligned fungal species including, *Botrytis cinerea* (Bot), *Cercospora zeae-maydis* (C.), both copies of *Cercospora zeina* (Cerc and Cerc_1), *Dissoconium acicular* (Dis)*, Fusarium graminearum* (Fus), *Sclerotinia sclerotiorum (Sc1), Sphaerulina musiva* (Sph), *Zymoseptoria ardabiliae* (Zymo) *and Zymoseptoria tritici* (Zymo_1 – Zymo_3). The figure shows a region within the amino acids sequences aligned to each other. Although the sizes of each protein is different, there are still conserved regions within each fungal protein.

Botrytis cinerea 14555
Sclerotinia sclerotiorum 3913
Fusarium graminearum 3612
Neurospora crassa 3123
Coccidioides immitis 7196
Viridothelium virens 213749
Delphinella strobiligena 376826
Aureobasidium pullulans 326261
Aureobasidium namibiae 10640
Aureobasidium melanogenum 41483
Aureobasidium subglaciale 64295

Dothideales

Zasmidium cellare 16833
Pseudocercospora fijiensis 104430
Sphaerulina musiva EMF08834.1
Cercospora zeae-maydis 43176
Cercospora zeina g860.t1
Dissoconium aciculare 313130
Teratosphaeria nubilosa 330419
Acidomyces richmondensis 3882
Piedraia hortae 1 255878
Baudoinia panamericana 46072
Elsinoe ampelina 267874

Capnodiales

Tothia fuscella 162235
Verruconis gallopava 123717
Verruconis gallopava 123718
Venturia inaequalis 21438
Trichodelitschia bisporula 527187

Venturiales

Aulographum hederae 452536
Saccharata proteae 35448
Aplosporella prunicola 350330
Phyllosticta citriasiana 419549
Macrophomina phaseolina 11790
Botryosphaeria dothidea 1 298165

Botryosphaeriales

Mytilinidion resinicola 503257
Lophium mytilinum 571245
Delitschia confertaspora 440147
Clohesyomyces aquaticus 565019
Lindgomyces ingoldianus 297995
Massariosphaeria phaeospora 569010
Trematosphaeria pertusa 558483
Karstenula rhodostoma 409053
Paraphaeosphaeria sporulosa OAG05184.1
Bimuria novae-zelandiae KAF1978011.1
Bimuria novae-zelandiae 525644
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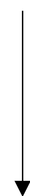
Byssothecium circinans 454834
Massarina eburnea 454415
Periconia macrospinosa 676255



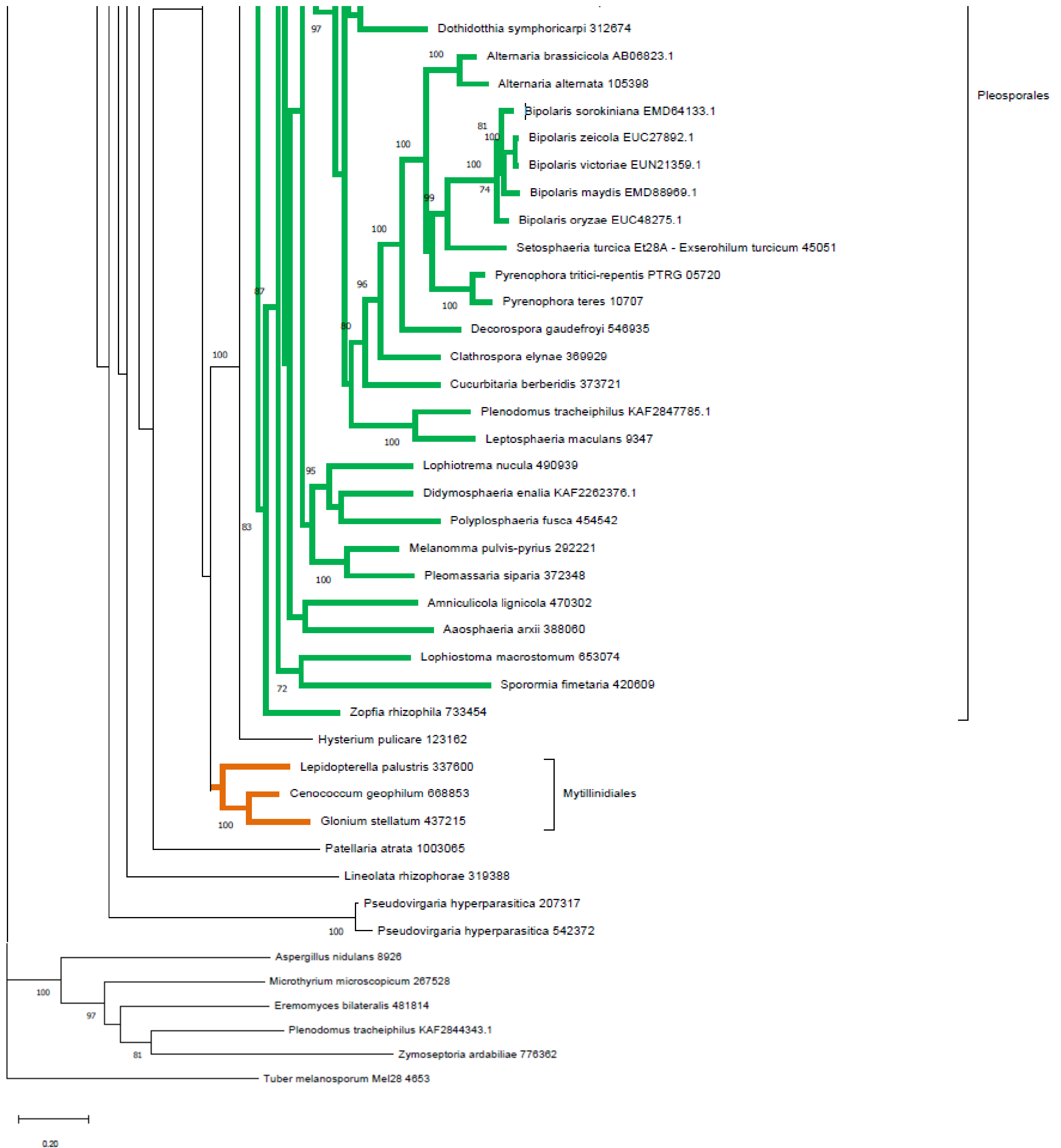
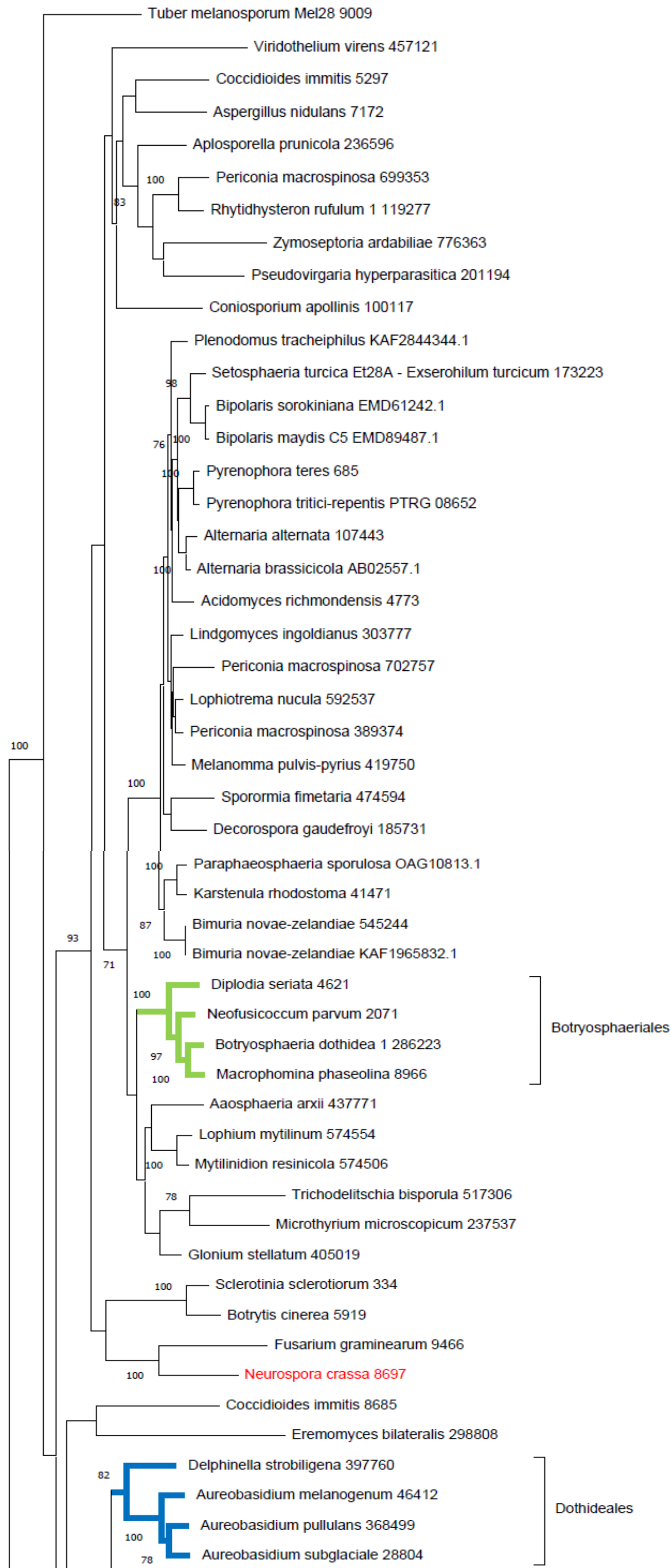


Figure S1.4: Phylogenetic tree of DCL-2 RAxML tree constructed from DCL-2 orthologs from species of Dothideomycetes, Leotiomycetes, Eurotiomycetes and Sordariomycetes. The tree was constructed using the Maximum Likelihood method based on 20 searches. Bootstrapping was done until convergence and values are shown at the nodes. *Neurospora crassa* the reference fungus is shown in red and *Cercospora zeina* which had a single ortholog for DCL-2 is shown in green.


Tuber melanosporum Mel28 9009
Viridothelium virens 457121
Coccidioides immitis 5297
Aspergillus nidulans 7172
Aplosporella prunicola 236596
Periconia macrospinosa 699353
Rhytidhysteron rufulum 1 119277
Zymoseptoria ardabiliae 776363
Pseudovirgaria hyperparasitica 201194
Coniosporium apollinis 100117
Plenodomus tracheiphilus KAF2844344.1
Setosphaeria turcica Et28A - Exserohilum turcicum 173223
Bipolaris sorokiniana EMD61242.1
Bipolaris maydis C5 EMD89487.1
Pyrenophora teres 685
Pyrenophora tritici-repentis PTRG 08652
Alternaria alternata 107443
Alternaria brassicicola AB02557.1
Acidomyces richmondensis 4773
Lindgomyces ingoldianus 303777
Periconia macrospinosa 702757
Lophiotrema nucula 592537
Periconia macrospinosa 389374
Melanomma pulvis-pyrius 419750
Sporormia fimetaria 474594
Decorospora gaudefroyi 185731
Paraphaeosphaeria sporulosa OAG10813.1
Karstenula rhodostoma 41471
Bimuria novae-zelandiae 545244
Bimuria novae-zelandiae KAF1965832.1
Diplodia seriata 4621
Neofusicoccum parvum 2071
Botryosphaeria dothidea 1 286223
Macrophomina phaseolina 8966
Botryosphaeriales
Aaosphaeria arxii 437771
Lophium mytilinum 574554
Mytilinidion resinicola 574506
Trichodelitschia bisporula 517306
Microthyrium microscopicum 237537
Glonium stellatum 405019
Sclerotinia sclerotiorum 334
Botrytis cinerea 5919
Fusarium graminearum 9466
Neurospora crassa 8697
Coccidioides immitis 8685
Eremomyces bilateralis 298808
Delphinella strobiligena 397760
Aureobasidium melanogenum 46412
Aureobasidium pullulans 368499
Aureobasidium subglaciale 28804
Dothideales
100
93
71
83
100
98
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100
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100
78
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82
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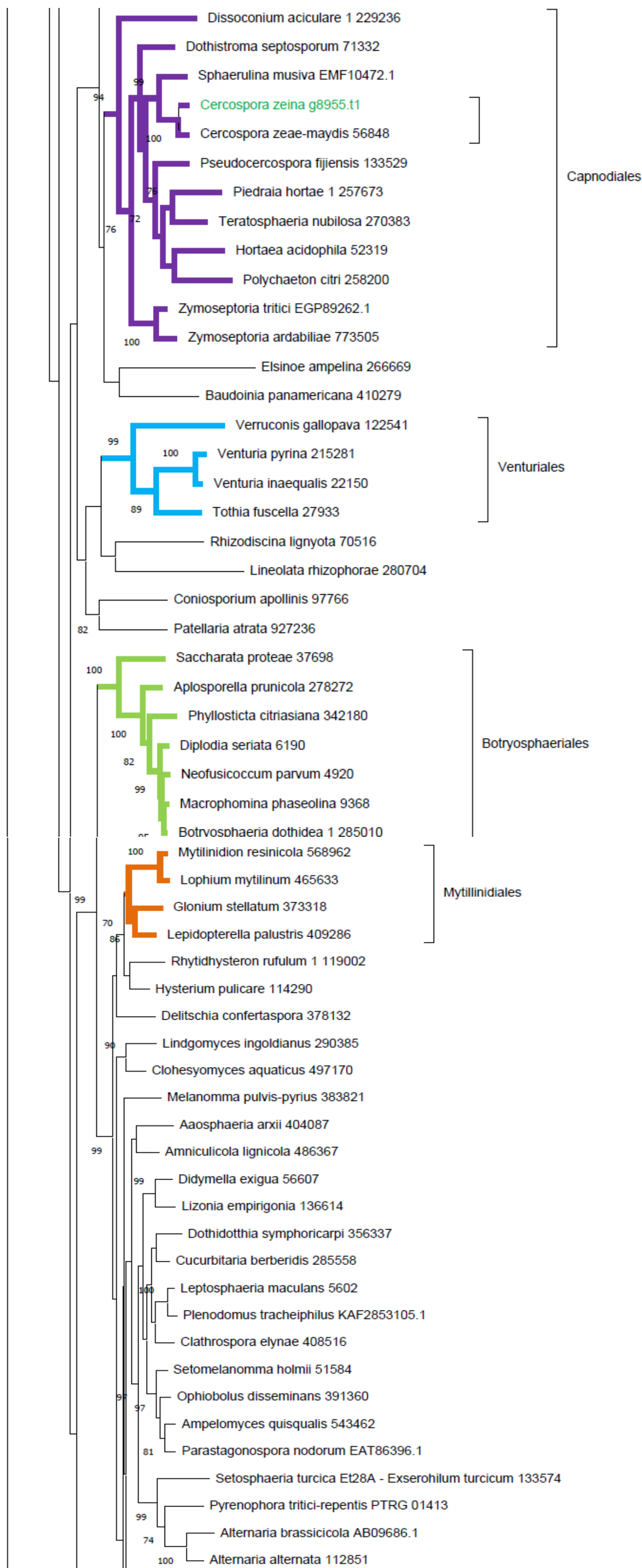
78



Dissoconium aciculare 1 229236
Dothistroma septosporum 71332
Sphaerulina musiva EMF10472.1
Cercospora zeina g8955.t1
Cercospora zeae-maydis 56848
Pseudocercospora fijiensis 133529
Piedraia hortae 1 257673
Teratosphaeria nubilosa 270383
Hortaea acidophila 52319
Polychaeton citri 258200
Zymoseptoria tritici EGP89262.1
Zymoseptoria ardabiliae 773505
Elsinoe ampelina 266669
Baudoinia panamericana 410279
Capnodiales
Verruconis gallopava 122541
Venturia pyrina 215281
Venturia inaequalis 22150
Tothia fuscella 27933
Venturiales
Rhizodiscina lignyota 70516
Lineolata rhizophorae 280704
Coniosporium apollinis 97766
Patellaria atrata 927236
Saccharata proteae 37698
Aplosporella prunicola 278272
Phyllosticta citriasiana 342180
Diplodia seriata 6190
Neofusicoccum parvum 4920
Macrophomina phaseolina 9368
Botryosphaeria dothidea 1 285010
Botryosphaeriales
Mytilinidion resinicola 568962
Lophium mytilinum 465633
Glonium stellatum 373318
Lepidopterella palustris 409286
Mytillinidiales
Rhytidhysteron rufulum 1 119002
Hysterium pulicare 114290
Delitschia confertaspora 378132
Lindgomyces ingoldianus 290385
Clohesyomyces aquaticus 497170
Melanomma pulvis-pyrius 383821
Aaosphaeria arxii 404087
Amniculicola lignicola 486367
Didymella exigua 56607
Lizonia empirigonia 136614
Dothidotthia symphoricarpi 356337
Cucurbitaria berberidis 285558
Leptosphaeria maculans 5602
Plenodomus tracheiphilus KAF2853105.1
Clathrospora elynae 408516
Setomelanomma holmii 51584
Ophiobolus disseminans 391360
Ampelomyces quisqualis 543462
Parastagonospora nodorum EAT86396.1
Setosphaeria turcica Et28A - Exserohilum turcicum 133574
Pyrenophora tritici-repentis PTRG 01413
Alternaria brassicicola AB09686.1
Alternaria alternata 112851

Lentithecium fluviatile 290147
Periconia macrospinosa 127448
Byssothecium circinans 504150
Massarina eburnea 478314
Trematosphaeria pertusa 109757
Massariosphaeria phaeospora 487212
Lophiostoma macrostomum 607168
Sporormia fimetaria 470298
Pleomassaria siparia 386448
Westerdykella ornata 397935
Didymosphaeria enalia KAF2261991.1
Polyplosphaeria fusca 574916
Lophiotrema nucula 500687
Aulographum hederae 399909
Pseudovirgaria hyperparasitica 133029
Massariosphaeria phaeospora 554523
Massarina eburnea 477389
Alternaria alternata 112300
Cucurbitaria berberidis 428875
Parastagonospora nodorum EAT81938.2
Lophiostoma macrostomum 713341
Sporormia fimetaria 331650
Trematosphaeria pertusa 517937
Paraphaeosphaeria sporulosa OAG09579.1
Karstenula rhodostoma 438576
Pleomassaria siparia 533008
Leptosphaeria maculans 2623
Plenodomus tracheiphilus KAF2851686.1
Cucurbitaria berberidis 305393
Ampelomyces quisqualis 446330
Ophiobolus disseminans 458662
Parastagonospora nodorum EAT81940.2
Setomelanomma holmii 117598
Byssothecium circinans 553556
Trematosphaeria pertusa 603685
Hysterium pulicare 115335
Lophiotrema nucula 656661
Massarina eburnea 537811
Cucurbitaria berberidis 429046
Pleomassaria siparia 404988
Melanomma pulvis-pyrius 395502
Massariosphaeria phaeospora 612925
Parastagonospora nodorum EAT81815.2
Bipolaris sorokiniana EMD58843.1
Bipolaris maydis C5 EMD90548.1
Bimuria novae-zelandiae 487177
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Bimuria novae-zelandiae KAF1972556.1
Bimuria novae-zelandiae 160517
Dothidotthia symphoricarpi 407140
Pyrenophora tritici-repentis PTRG 09994
Pyrenophora teres 9155
Alternaria brassicicola AB00206.1
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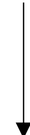

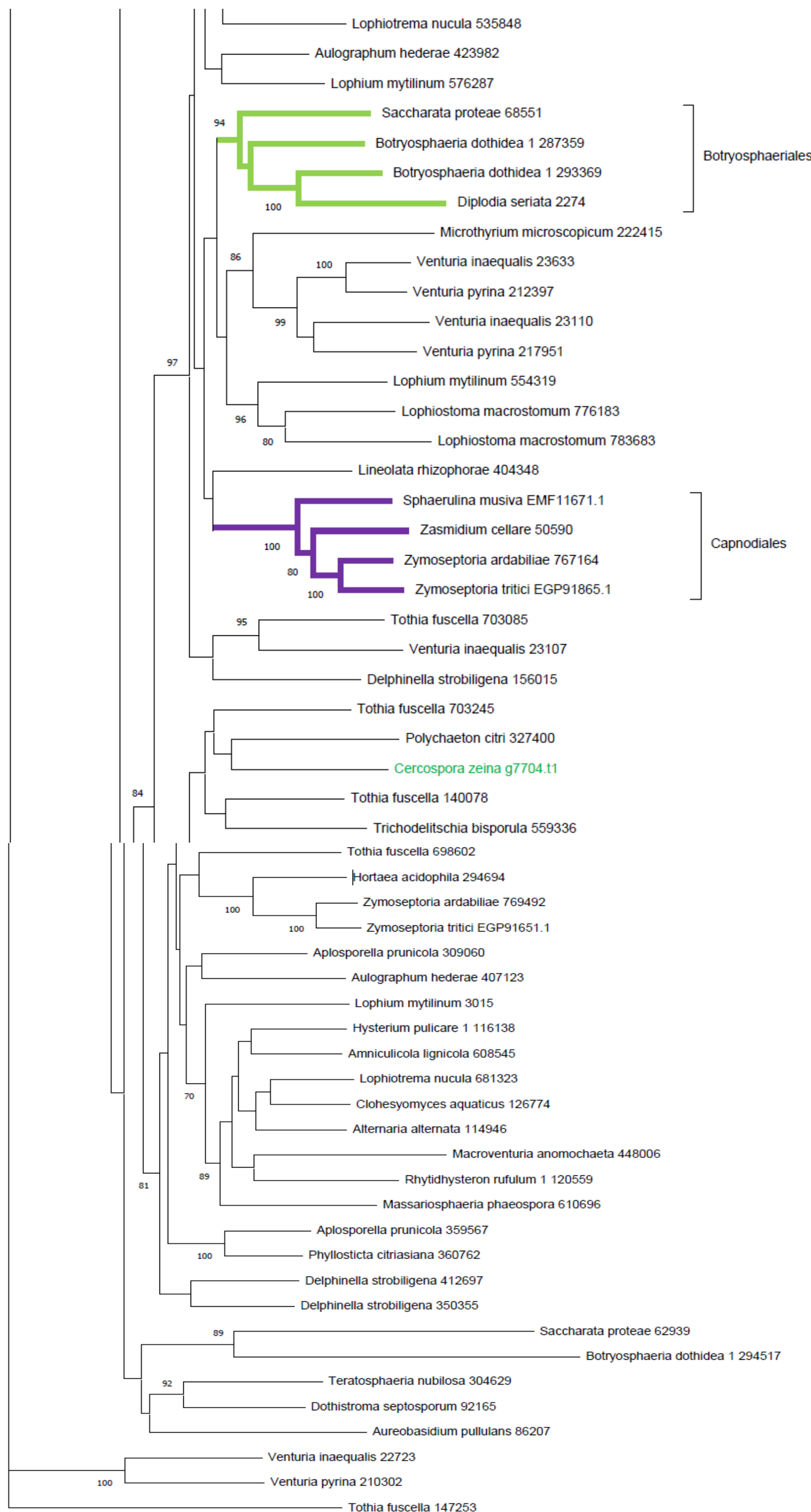

Figure S1.5: Phylogenetic tree of QDE-2 RAxML tree constructed from QDE-2 orthologs from species of Dothideomycetes, Leotiomycetes, Eurotiomycetes and Sordariomycetes. The tree was constructed using the Maximum Likelihood method based on 20 searches. Bootstrapping was done until convergence and values are shown at the nodes. *Neurospora crassa* the reference fungus is shown in red and *Cercospora zeina* which had two orthologs for QDE-2 is shown in green

Appendix B

Supplementary Tables

Table S2.1 Primers used in this study

Gene target	Name and Primer pair (5'-3')	Tm (°C)	cDNA/gDNA Expected size (bp)
Green fluorescent protein gene	T7-GFP F : TAATACGACTCACTATAGGGCAAGGAGGACGGCAACATC	59	365/365
	T7-GFP R : TAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGC	59	
	RT GFP F : TCAAGATCCGCCACAACATC	59	115/115
	RT GFP R : GTGCTCAGGTAGTGGTTGTC	59	
C. zeina Chitin synthase D gene	CSD F: CATGACCGACTACCCAAATAG	55	798/798
	CSD R: TACAAGTCAGCACCACCAACCGACGACGTTCTCCGAATAAT	55	
	RT CSD F: CTGCCATCAGTTTCACCATTTA	59	93/146*
	RT CSD R: CGAGCAGGATAAGAGGAATGAC	59	
C. zeina Phosphatidyl serine decarboxylase gene	PSD F: GAGCTACAACCCATCGAACTAGGCGTATACCAGGCATTCCTATC	56	404/404
	PSD R: GAAGCCCTCGACATTGACAT	56	
	RT PSD F: TCGTGAAGATGTTTGAGGAAGT	58	82/132*
	RT PSD R: GTGGTAGTCGCGGATTTGT	58	
C. zeina ECP2 effector protein gene	ECP2 F: ATTATTCGGAGAACGTCGTCGGTTGGTGGTGCTGACTTGTA	56	201/201
	ECP2 R: GATAGGAATGCCTGGTATACGCCTAGTTCGATGGGTTGTAGCTC	56	
	RT ECP F: GTCCCACAGAGGAAGAATG	59	137/137
	RT ECP R: TTGTCGAGGCTCTGAATG	59	
Entire 3 gene construct (3Cz_dsRNA)	T7-CSD F: TAATACGACTCACTATAGGGCCATGACCGACTACCCAAATAG	72	1443/1443
	T7-PSD R: TAATACGACTCACTATAGGGGAAGCCCTCGACATTGACAT	72	

C. zeina Glyceraldehyde-3-Phosphate Dehydrogenase gene	GAPDH F: TTCATCGAGCCACACTAC GAPDH R: TGACGATCAGGCCTTTG	59 59	103/161*
C. zeina 40S Ribosomal protein gene	40S F: GGTCCTCAAGGTCATTCTC 40S R: TTGACACCCTTTCCAGTC	59 59	102/102

*Can be used to check for gDNA contamination.

- Single underlined sequences: T7 promoter sequences
-- Double underlined sequences: Overlapping sequence areas

Table S2.2 C. zeina reference gene stability after dsRNA treatments
This table summarizes the geNorm expression stability values of the reference genes.

Reference target	Gene stability (M)*	Pair wise variation (CV)*
GAPDH	0.353	0.121
40S	0.353	0.123
Average	0.353	0.122

*Reference gene is suitable for expression normalization when: M- value is < 0.5 and CV values is < 0.25 is desired.

Table S2.3 Each gene and their respective regression curve equation**, c**orrelation coefficient (R^2) as well as the efficiency (E) of each genes curve is shown below.

Gene	Equation of regression line	R^2	Efficiency
*40S**	y = -3.10x + 21.37	0.97	1.10
*GAPDH**	y = -3.08x +17.55	0.98	1.11
ChsD	Y= -3.29 + 16.93	0.98	1.02
Psd3	Y = -3.77x +16.57	0.95	0.84
Gfp	y = -3.09x + 15.91	0.97	1.10
Ecp2	Y = -3.20 + 21.20	0.95	1.05

*Reference genes used showed the best PCR efficiency.

Table S2.4 Two-way ANOVA tables from GFP fluorescence measured after different concentrations of dsRNA was added at different time points.

Source of Variation	**% of total variation**	**P value**		
Interaction (concentration x time)	8,55	0,02		
Concentration	58,98	< 0.0001		
Time	0,71	0,51		
Source of Variation	**P value summary**	**Significant?**		
Interaction	*	Yes		
Concentration	****	Yes		
Time	ns	No		
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	6	1,14E+12	1,91E+11	2,69
Concentration	3	7,89E+12	2,63E+12	37,15
Time	2	9,56E+10	4,78E+10	0,68
Residual	60	4,248E+12	7,08E+10	

Table S2.5 RNAseq data set for C. zeina target genes that were chosen (GEO dataset: GSE94442 and GSE90705). Target genes for external RNAi were selected based on relatively high in planta expression (log2CPM > 2) and in vitro expression (cornmeal agar counts > 800)

			In planta					In vitro	
Genbank Protein	**Gene Model name^**	**Annotation (Genbank)**	**B73 upper leaf, low Cz infection (average CPM)**	**B73 lower leaf, high Cz infection (average CPM)**	**Log2FC**	**Log 2CPM#**	**FDR adjusted PValue**	**Cz in vitro, Cornmeal-agar, counts**	**Cz in vitro, V8 agar, counts**
PKS02949.1	czeina9g0 02110	Chitin synthase D	4 ± 4.6	625 ± 114	7.32	4.2	6E-66	10787	5210
PKS00696.1	czeina23g 001390	Phosphatidylserine decarboxylase proenzyme 3	3 ± 3.2	1696 ± 319.1	9.02	5.6	9E-103	1852	4364
PKR94769.1	czeina239 g000040	ECP2	368 ± 246.1	2740 ± 349.6	3.55	6.4	3E-03	10451	207
PKR99485.1 (for reference)	czeina34g 000660	Cryparin*	297 ±	13065	5.68	8.7	3E-06	21727	668
For reference	All 8852 *C.zeina* genes	90th percentile	5	422	8.5	3.6	1E-44	4619	4183
For referenceAll 8852 genes in datasets	All 8852 *C.zeina* genes	Average	3	229	6.7	1.3	2E-04	2431	2408

RNAseq data obtained for 8852 *C. zeina* genes
^ Gene model names in Genbank all have prefix: BST61_
* in planta: most highly expressed C.zeina gene with fungal annotation
# log2CPM = log2 (average CPM of *C.zeina* gene in B73 upper and lower leaves)

Table S2.6 Summary of *C. zeina* genes chosen for RNAi, their roles and where they have been used previously.

Gene target	Role Function of gene product in fungal growth or pathogenicity	References to support choice of gene as a target for gene knockdown
Chitin synthase D gene (*ChsD*)	Responsible for the synthesis of chitin (an important polysaccharide component of the fungal cell wall)	Chitin also plays a role in immune responses against fungi (Lenardon et al., 2010) *F. graminearum* chitin synthase b targeted by HIGS (Cheng et al., 2015) *S. sclerotiorum* chitin synthase targeted by HIGS (Andrade et al., 2016)
Phosphatidyl serine decarboxylase pro-enzyme 3 gene (*Psd3*)	Catalysis the formation of phosphatidylethanolamine (PtdEtn) a major component of cell membranes	Phosphatidylserine synthesis is essential for viability of the human fungal pathogen *Cryptococcus neoformans* (Cassilly and Reynolds, 2018; Konarzewska et al., 2019)
Extracellular protein 2 gene (*Ecp2*)	Induces necrosis in plants, increasing the pathogenicity of the fungus on its host	ECP2 is an important virulence factor (Laugé et al., 1997; Stergiopoulos et al., 2010)

Supplementary Figures

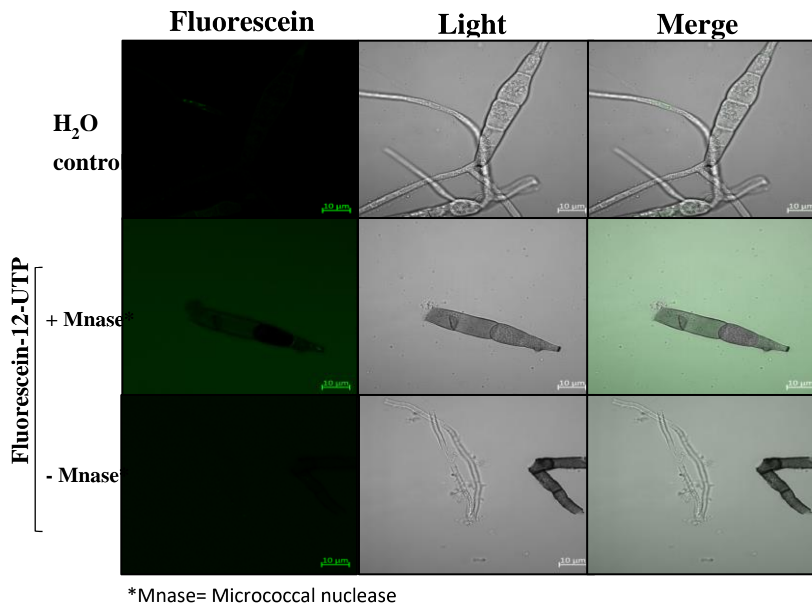

Figure S2.1: Confocal fluorescence microscopy depicting dsRNA uptake by wild-type *C. zeina* conidia - Negative controls. LSM 880 confocal microscopy was used to observe fluorescence at 100x magnification. Fluorescein-12-UTP wavelength at 495/525 nm was used. Water-treated samples showed no fluorescence and Fluorescein-12-UTP-treated samples showed no fluorescence inside the cells.

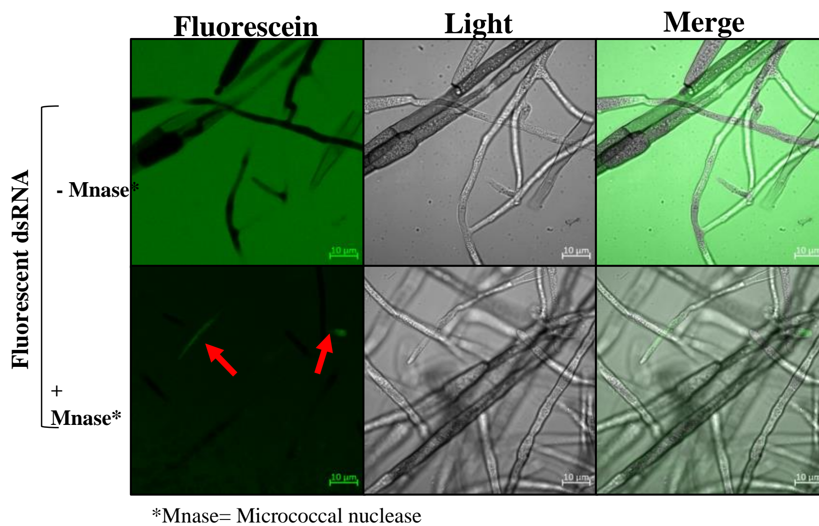

Figure S2.2: Confocal fluorescence microscopy depicting fluorescent dsRNA uptake by wild type *C. zeina* conidia. LSM 880 confocal microscopy was used to observe fluorescence at 100x magnification. The fluorescent dsRNA was observed using a wavelength of 495/525 nm was used. Small amount of RNA uptake was observed within the conidial cells after micrococcal nuclease treatment, indicated with the red arrows.

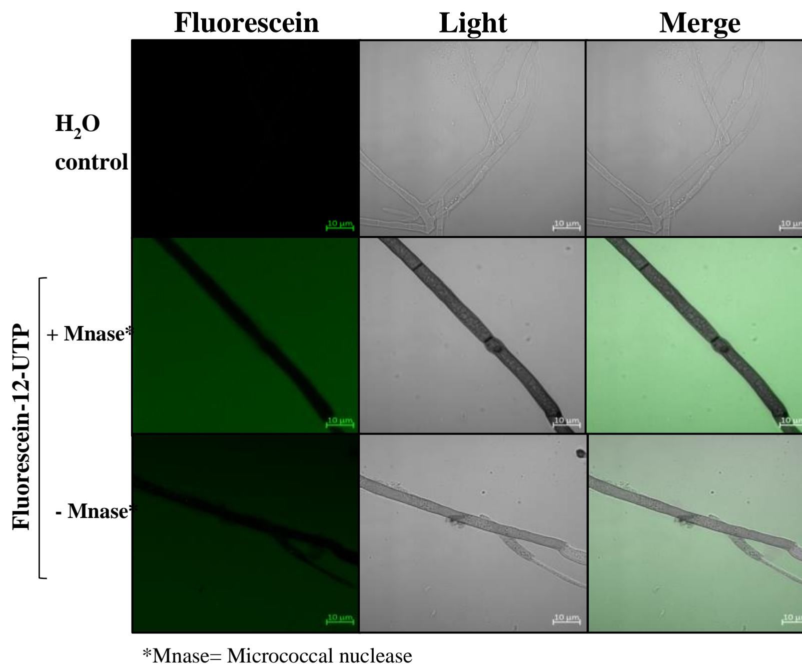

Figure S2.3: Confocal fluorescence microscopy indicating RNA uptake by wild type *C. zeina* mycelia - Negative controls. LSM 880 confocal microscopy was used to observe fluorescence. Fluorescein-12-UTP wavelength at 495/525 nm was used. Water-treated samples showed no fluorescence. The Fluorecein-12-UTP-treated samples showed no internal fluorescence before or after treatment with micrococcal nuclease.

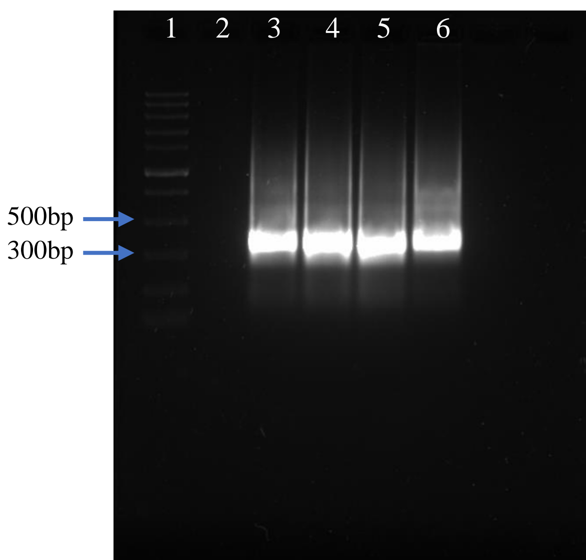

Figure S2.4: Gel electrophoresis showing GFP_dsRNA final product after in vitro transcription. Lane 1 contains the Fast DNA ladder (NEB), lane 2 is blank, lane 3-6 contains 4 different dsRNA transcription products made in parallel. The GFP dsRNA expected size was 325 bp, which is shown on this gel.

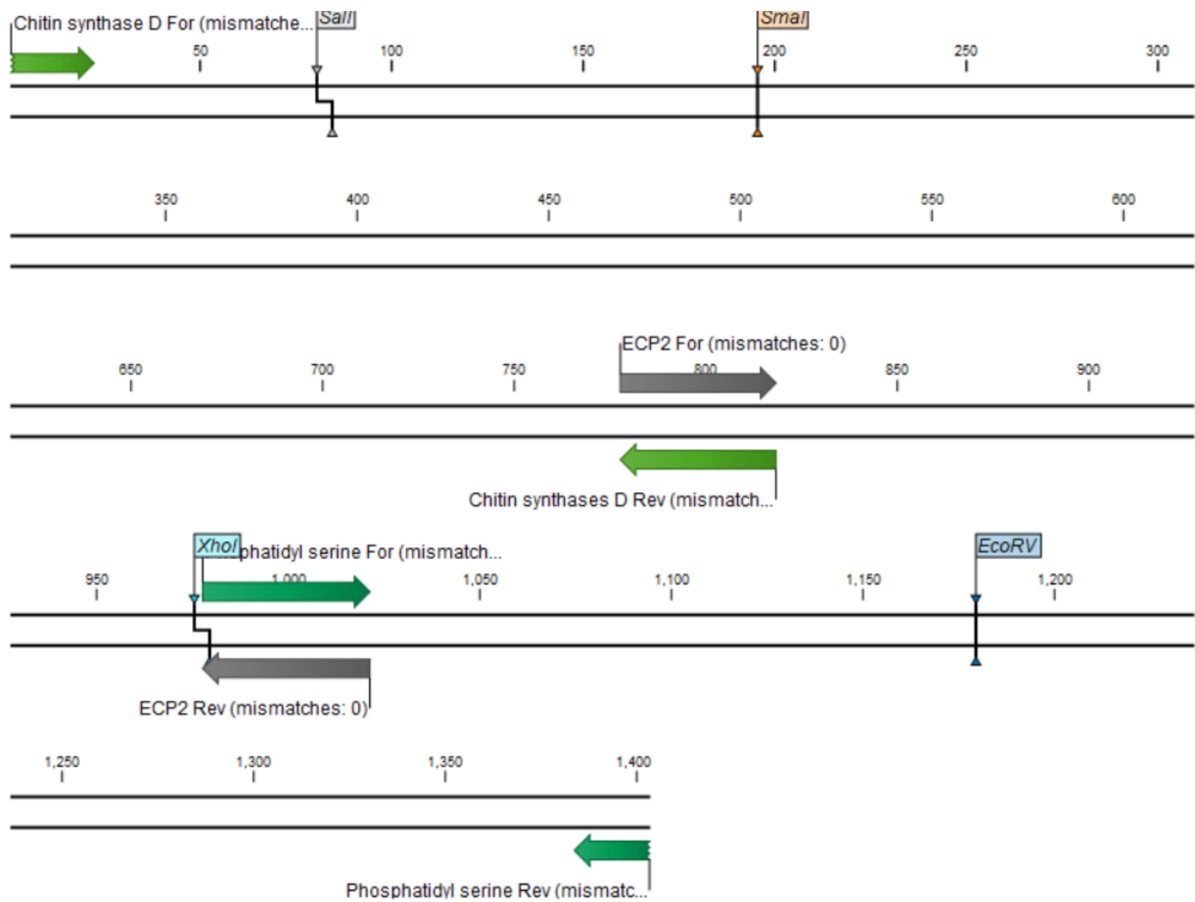

Figure S2.5: Graphical representation of the 3Cz_dsRNA construct which was created. This construct targeted a 798 bp fragment of Chitin synthase D, 201 bp fragment of ECP2 and 404 bp fragment from Phosphatidyl serine decarboxylase, creating a final dsRNA construct of 1403 bp

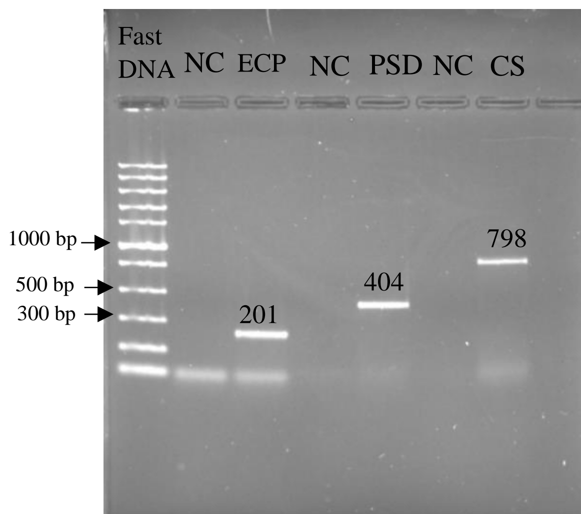

Figure S2.6: Amplification of each *C. zeina* gene product separately. Fast DNA ladder (NEB) was used. ECP2 product is shown in lane 3 containing the 201bp fragment. PSD3 is shown in lane 5 containing the 404 bp fragment. CHSD is shown in lane 7 containing the 798 bp fragment. The empty lanes are the negative controls

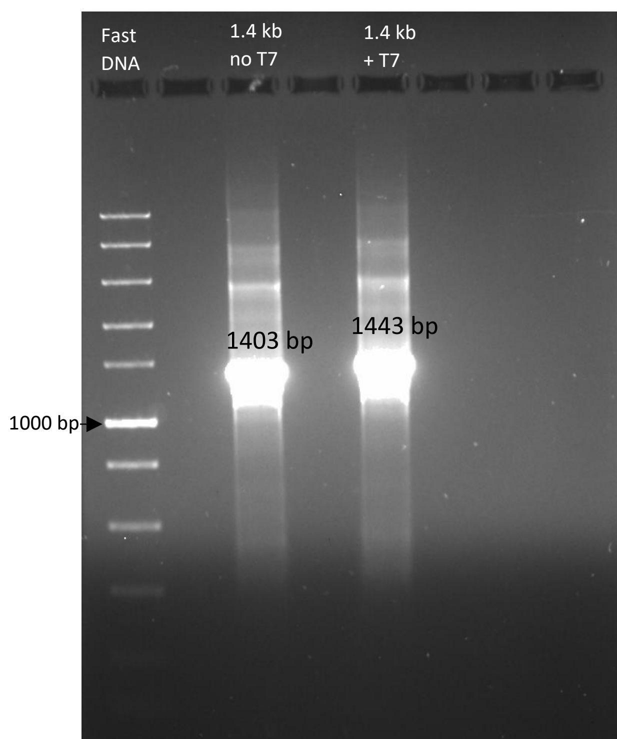

Figure S2.7: Amplification of all three gene products together. Fast DNA ladder (NEB) was used in lane 1. Lane 2 and 4 contain the negative controls. Lane 3 and 5 indicates the full-length amplicons after overlap PCR, using either primers containing T7 overhangs, or those without T7 overhangs. The expected product of 1.4 kb was obtained

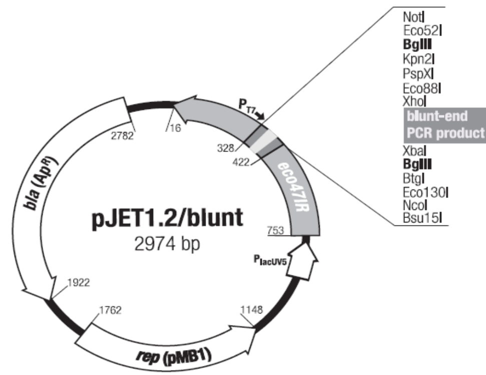

Figure S2.8: pJET1.2/blunt vector plasmid map. The 1.4 kb plasmid was cloned into the pJET1.2/blunt vector.

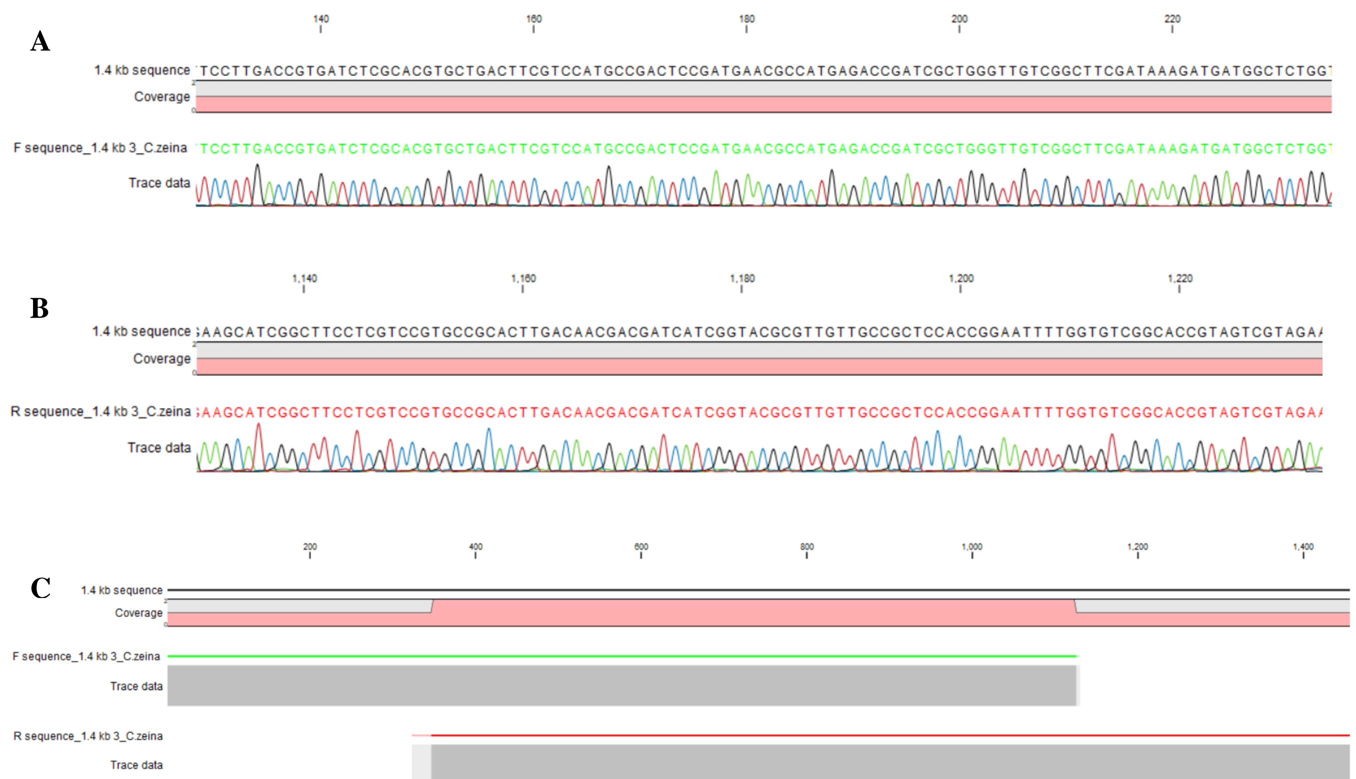

Figure S2.9: Sequencing of the 1.4kb product cloned into the pJET vector. A. The DNA sequence result obtained by using the forward primer and aligning it to the 1.4 kb expected sequence (zoomed in). B. The DNA sequence result obtained by using the reverse primer and aligning it to the 1.4 kb expected sequence fragment (zoomed in). C. The whole contig created after aligning both sequence results to the 1.4 kb expected sequence (zoomed out). The black line indicates the expected sequence, the green line indicates the forward sequencing strand, the red line indicates the reverse sequencing strand. The pink areas indicate where the DNA sequence aligns to the expected 1.4 kb fragment, and the thicker pink area indicates where both sequencing products overlap and also aligns to the 1.4 kb fragment.

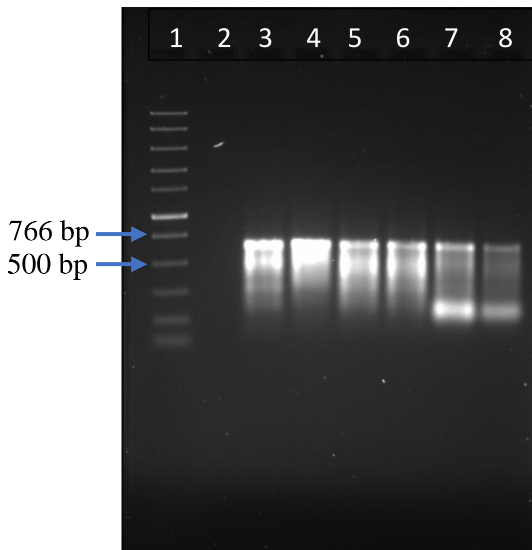

Figure S2.10: Double-stranded RNA targeting 3 *C. zeina* genes, shown on 1% agarose gel (3Cz_dsRNA). Lane 1 contains the Fast DNA ladder (NEB), lane 3-8 contains the different dsRNA fragments created after transcription in parallel. The expected bands were 1.4 kb in length, but only a 750 bp fragment could be observed possibly due to the difference in migration between dsRNA and dsDNA (Livshits et al., 1990).

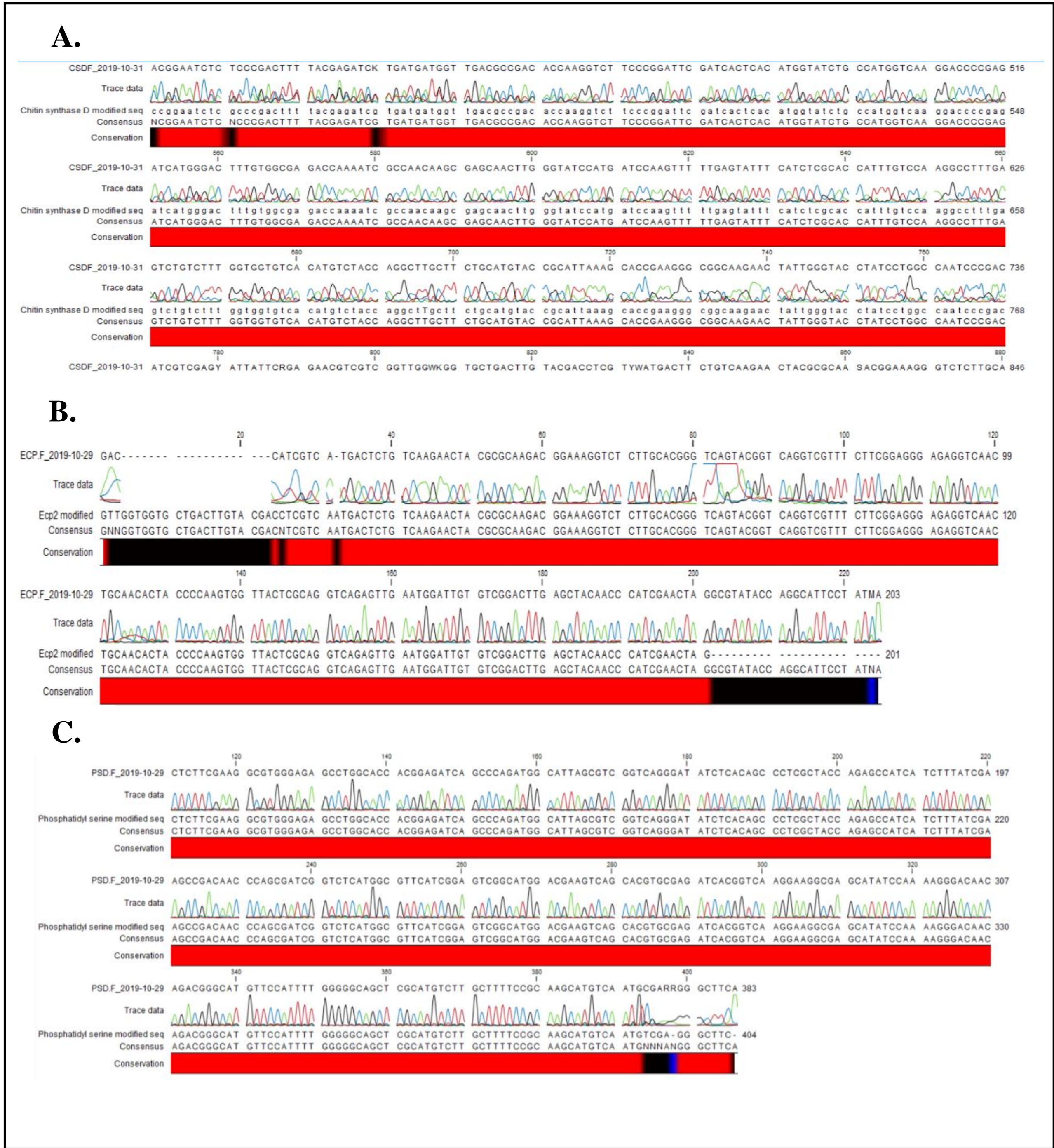

Figure S2.11: Sequencing of the dsRNA product (cDNA synthesis), using gene specific primers and alignment to specific gene targets. A) *ChsD* target gene region within dsRNA aligned to a 350 bp region of the *ChsD,* indicating that only a part of this gene was targeted by the dsRNA B) *Ecp2* target gene region within dsRNA sequence aligned to the full *Ecp2* target region C) *Psd3* target region within dsRNA sequence aligned to the full *Psd3* target region

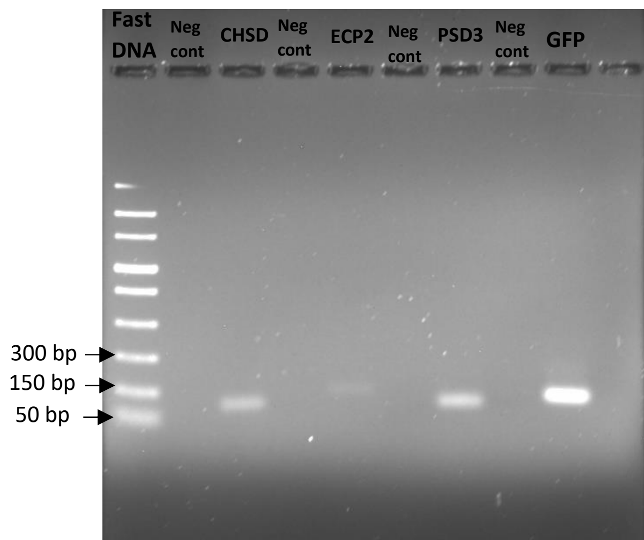

Figure S2.12: RT-PCR analysis of *C. zeina* genes. After cDNA synthesis of the total *C. zeina* RNA, a pool of all the cDNA samples (*C. zeina* treated and untreated with dsRNA) were used for RT-PCR. Lane 1 is the Fast DNA ladder, lane 2, 4, 6 and 8 contain the negative controls. Lane 3 contains the *ChsD* amplicon (93 bp), lane 5 contains the *Ecp2* amplicon (137 bp), lane 7 contains the *Psd3* amplicon (82 bp) and lane 9 contains the *Gfp* amplicon (115 bp). Fast DNA ladder (New England Biolabs) was used.

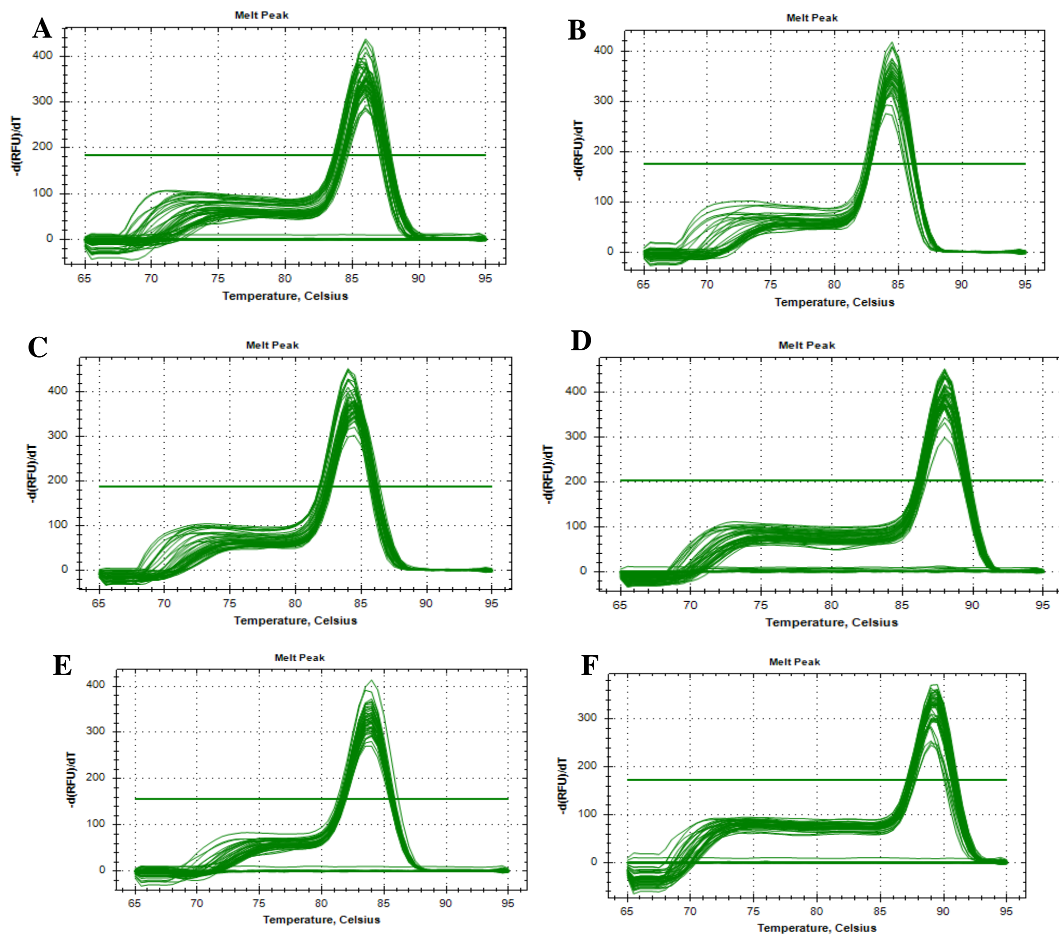

Figure S2.13: Melting curves from RT-qPCR analysis of *C. zeina* reference and target genes. The melting curves of the *C. zeina* target genes (*ChsD*, *Ecp2*, *Psd3* and *Gfp*) and the two stable reference genes (*GAPDH* and *40S*) are shown here, plotted as the negative rate of change in relative fluorescence units (RFU), as the temperature (T) is changed. The order of gene melting curves represented above are as follows: A-*GAPDH*, B-*40S*, C- *ChsD*, D- *Ecp2*, E- *Psd3*, F- *Gfp*. The analysis of the melting curves indicated single peaks for each analysed gene, indicating specific amplification products without any primer dimers. The melting temperatures of all the genes mentioned were 85.5 °C, 84.5 °C, 84.5 °C, 88 °C, 84 °C, 89.5 °C.

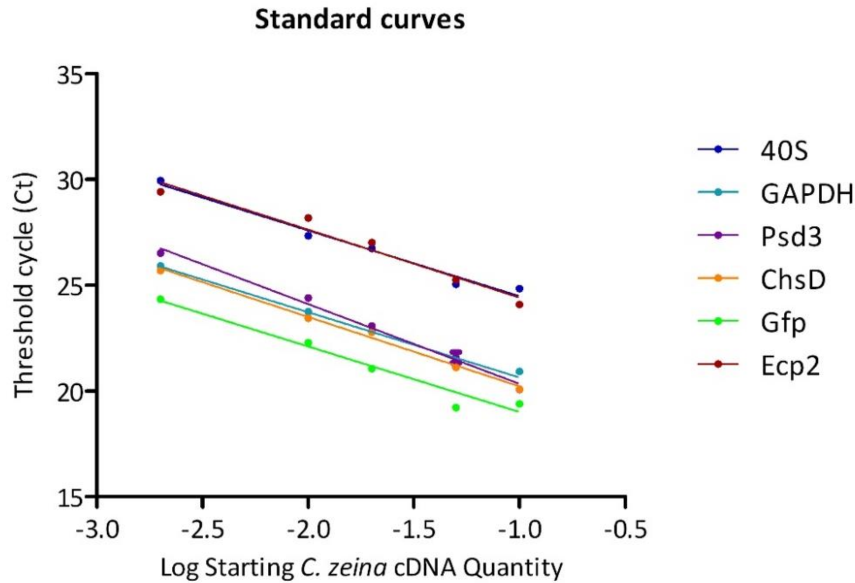

Figure S2.14: Standard curves generated for *C. zeina* reference and target genes. A cDNA pool isolated from the transgenic fungus treated both with RNA and with water was used to generate the standard curve of each gene. The standard curves for the reference genes *GAPDH* and *40S* as well as the target genes, *Psd3*, *ChsD*, *Ecp2* and *Gfp* were generated by plotting the threshold cycle (Ct) value to the log of the *C. zeina* cDNA starting concentration. A linear regression trend line was drawn between data points of each gene. PCR efficiencies of each gene is given in Table S2.

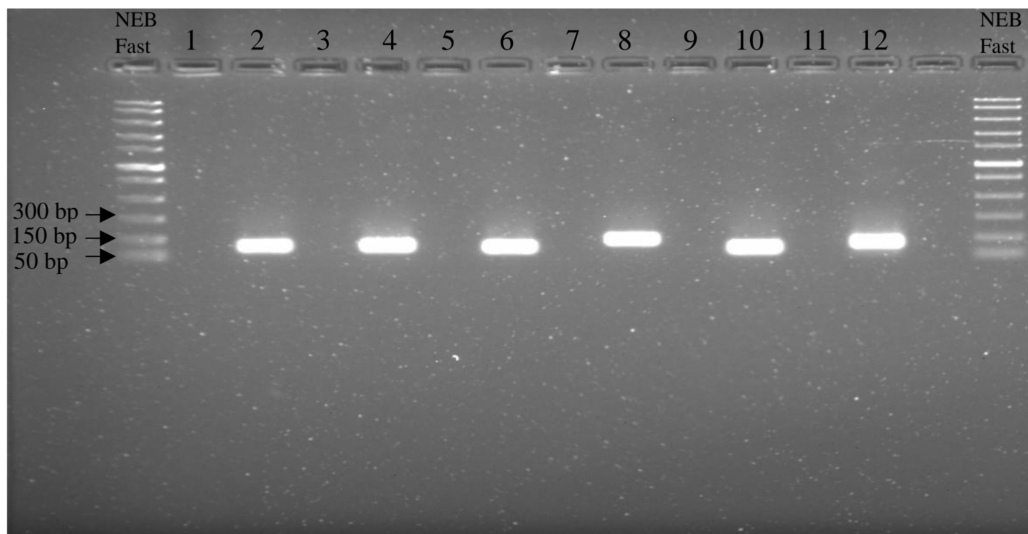

Figure S2.15: Amplification specificity of *C. zeina* RT-qPCR products. Selected RT-qPCR products were analysed on a 2% Agarose gel. Negative controls represent the RT-qPCR products without any cDNA template. Lanes: (1) negative control (2) *40S* (102 bp), (3) negative control, (4) *GAPDH* (103 bp), (5) negative control, (6) *ChsD* (93bp), (7) negative control, (8) *Ecp2* (137bp), (9) negative control, (10) *Psd3* (82 bp), (11) negative control, (12) *Gfp* (115 bp). Fast DNA ladder (New England Biolabs) was used.