Gene silencing mediated by dsRNA reduces grey leaf spot disease in maize

by

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Declaration

I, Carla Buitendag, declare that the dissertation, 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: 13 June 2023

Summary

Grey leaf spot (GLS) is a devastating fungal foliar disease which affects maize globally. The causal agent for GLS in southern Africa is *Cercospora zeina*. Current methods to control GLS include chemical fungicides and tilling. However, GLS remains a destructive disease with up to 60% yield losses reported in susceptible hybrids. Advances in plant biotechnology utilize a natural gene silencing mechanism known as RNA interference (RNAi). RNAi is mediated by double-stranded RNA (dsRNA) that targets the expression of genes that share sequence complementarity with the dsRNA. This phenomenon has been adapted in new crop protection strategies such as spray-induced gene silencing (SIGS), which involves the external application of dsRNA that targets pathogenicity genes to combat disease. SIGS is a gene-specific, environmentally friendly, biodegradable, and safe treatment. In a previous study from our research group a 3Cz dsRNA construct was generated which targets three pathogenicity genes in *C. zeina*: *chitin synthase D (CHSD), phophatidylserine decarboxylase proenzyme 3 (PSD3),* and *extracellular protein 2 effector* (*ECP2)*. The *in vivo* results indicated that 3Cz dsRNA significantly reduced the fungal cell viability by 34% ($\alpha = 0.05$). In this study, the aim was to test the ability of 3Cz dsRNA to control GLS disease *in planta*. Maize leaves were inoculated with *C. zeina* conidia and treated with 3Cz dsRNA or a negative treatment control, Green fluorescent protein (GFP) dsRNA. The dsRNA treatments were either applied at the same time as fungal inoculation or 24 h later. In addition, the percentage of germinated conidia over a 24-h period was determined *in vitro* with light microscopy (LM) and *in planta* using scanning electron microscopy (SEM). This study demonstrated that the 3Cz dsRNA construct significantly reduced GLS disease symptoms by 39 – 56% when applied at 1 day post inoculation (dpi), compared to the positive disease control ($\alpha = 0.05$). The GFP dsRNA treatments resulted in similar GLS disease symptoms as the positive disease control. Interestingly, the 3Cz dsRNA application did not significantly reduce GLS symptoms when applied at the same time as fungal inoculation, compared to the positive disease control and GFP dsRNA treatments. The LM and SEM results indicated that up to 77% of the *C. zeina* conidia germinated within 24 h *in vitro* and *in planta*. It is hypothesised that germinated conidia allowed for more efficient uptake of dsRNA, which could explain why 3Cz dsRNA application at 1 dpi significantly reduced GLS disease symptoms. A new dsRNA construct was created based on previous successful SIGS studies in *Botrytis cinerea*, which had demonstrated efficient disease control by targeting three genes involved in vesicle-trafficking pathways. The new dsRNA designed in this study will be evaluated in future studies with the aim to obtain highly effective SIGS results in *C. zeina* which produce almost no GLS symptoms in maize. This study demonstrates promising results that RNAi technologies such as SIGS could be used to control GLS disease in maize.

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List of Abbreviations

3Cz dsRNA	dsRNA construct that targets three genes in *C. zeina*
AGO	Argonaute
AUDPC	area under disease progress curve
BLAST-p	Basic Local Alignment Search Tool; protein-protein search
CHSD	Chitin synthase D
DCL	Dicer-like
DCTN1	Dynactin 1
ddH_2O	double-distilled water
disiRNA	Dicer-independent siRNA
DLDM	disiRNA loci DNA methylation
DMI	demethylase inhibitors
dsRNA	double-stranded RNA
ECP2	Extracellular protein 2 effector
esRNA	endogenous sRNA
EtBr	Ethidium bromide
ex-siRNA	exon-derived regulatory siRNA
F	forward
GFP	Green fluorescent protein
GM	genetically modified
GMO	genetically modified organism
GLS	grey leaf spot
HIGS	host-induced gene silencing
HMDS	hexamethyldisilazane
HYL1	Hyponastic leaves 1
LM	light microscopy
miRNA	micro RNA
milRNA	microRNA-like small RNA
mRNA	messenger RNA
MSUD	meiotic silencing by unpaired DNA
natsiRNA	natural-antisense siRNA
ncRNA	non-coding RNA
pri-miRNA	primary miRNA
PSD3	Phophatidylserine decarboxylase proenzyme 3
qde	quelling-defective
QIP	QDE-2 interacting protein
qiRNAs	QDE-2-associated sRNAs
R	reverse
rdRNA	RDRP-dependent degraded RNA
RDRP	RNA-directed RNA polymerase
RNAi	RNA interference
RIP	repeat-induced point mutations
RISC	RNA-induced silencing complex
RITS	RNAi-induced transcriptional silencing complex
SAC1	Suppressor of actin 1
SAD-1	Suppressor of ascus dominance 1
SEM	scanning electron microscopy
SIGS	spray-induced gene silencing
siRNA	small interfering RNA
SMS-2	Suppressor of meiotic silencing 2
sRNA	small RNA
ssRNA	single-stranded RNA
TAE	Tris-acetate-EDTA
tasiRNA	*trans*-acting siRNA
Tm	melting temperature
VIGS	virus-induced gene silencing
VPS51	Vacuolar protein sorting 51
USA	United States of America

List of Units

°C	degrees Celsius
µg	microgram
µL	microlitre
µM	micromolar
bp	base pair
cm	centimetre
dpi	days post inoculation
g	gram
h	hour
kb	kilobase
M	molar concentration
min	minute
mL	millilitre
mm	millimetre
ng	nanogram
nt	nucleotide
RH	relative humidity
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight

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Preface

The increasing human population is putting a considerable strain on the world's food security. The agricultural sector is constantly facing yield limitations due to crop pathogens that are difficult to manage (Savary et al., 2019). Maize is among one of the most important cereals globally and is a staple food in many countries. Grey leaf spot (GLS) is a devastating foliar disease of maize and is caused by the fungal *Cercospora* species. *Cercospora zeina* is responsible for GLS disease occurring in sub-Saharan Africa (Ward et al., 1999). GLS disease reduces the crop yield and product quality, and the disease is difficult to control due to the persistence of the fungal conidia in soil, which can overwinter in the crop residue, and spread through rain splash and water movement (Ward, 1996).

Novel crop protection studies are adapting natural RNA interference (RNAi) pathways as a means to control plant pathogens (Salame et al., 2011, Baulcombe, 2015). One of these technologies is known as spray-induced gene silencing (SIGS) and utilises the application of external dsRNA which facilitates the degradation of target gene transcripts that bear complementary sequences to the exogenous dsRNA. Several recent studies in SIGS have successfully reduced disease severity and infection in various fungal pathogens, such as *Fusarium graminearum*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum* (Koch et al., 2016, Wang et al., 2016, McLoughlin et al., 2018). These SIGS studies demonstrate an efficient yet environmentally safe alternative to harmful chemical fungicides. An additional advantage of external dsRNA technologies is that the treatment is non-transgenic and does not require genetically modified crops to obtain an environmentally friendly and safe product (Arpaia et al., 2021).

The Molecular Plant-Pathogen Interactions (MPPI) research group at the University of Pretoria in South Africa has a particular interest in *C. zeina* studies. A previous Master's research study produced from MPPI created a single dsRNA construct named 3Cz dsRNA (Marais, 2020). 3Cz dsRNA targets three pathogenicity genes in *C. zeina*; a gene involved in chitin synthesis (*CHSD*), a gene involved in the biosynthesis of a major cell membrane component (*PSD3*), and an effector gene (*ECP2*). This 3Cz dsRNA construct was tested *in vitro* and the study demonstrated that not only does *C. zeina* have the ability to efficiently take up external dsRNA (Marais, 2019), but that 3Cz dsRNA reduced the relative gene expression of target genes, and significantly reduced the fungal cell viability by 34% when applied to *C. zeina* cultures ($\alpha = 0.05$) (Marais, 2020).

This study was undertaken with the aim to test the ability of the 3Cz dsRNA to control GLS disease in maize leaves that have been inoculated with *C. zeina* conidia. The application of the construct was tested at two timepoints; immediate application in conjunction with the conidia inoculation, and 24 h after inoculation. Limited histological research is available on *C. zeina* structures soon after their inoculation on maize leaves. Thus, this study investigated how soon *C. zeina* conidia germinate *in vitro* and after inoculation on maize leaves. In addition, a new dsRNA construct was designed in this study with the aim to obtain even more significant GLS disease control results in future studies.

The research in this dissertation was performed in the laboratories of the MPPI research group at the Department of Plant and Soil Sciences, Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa.

The structure of this dissertation is in two chapters; the first chapter is a comprehensive literature review on the RNAi phenomenon and its applications in crop protection strategies, and the second chapter is the experimental chapter, written in publication format (for the journal *Frontiers in Microbiology*), and reports on the experiments involving external dsRNA to control GLS disease and *C. zeina* conidia germination studies.

Research outputs from this study:

Prof. Dave K. Berger, Ms Ingrid Marais, Ms Carla Buitendag, Dr. Tuan A. Duong, Dr Bridget G. Crampton, Prof. Jacques Theron. **RNAi "vaccines" for plants: grey leaf spot disease control in maize.** The South African Genetics Society & South African Society for Bioinformatics BIO2022 Conference; 2022 April 24-27; Stellenbosch, South Africa. (Oral presentation)

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Chapter 1

Literature Review: RNA interference and its application as a crop protection strategy

Abstract

Grey leaf spot (GLS) is a devastating foliar disease of maize and the causal agent for this disease in southern Africa is the fungal foliar pathogen *Cercospora zeina*. GLS is one of the most significant maize diseases in sub-Saharan Africa and the USA. Grain yield losses of up to 60% have been reported in susceptible maize hybrids in South Africa. GLS is conventionally treated with commercially available fungicides which pose dangerous health and environmental risks. The global food security crisis emphasizes the increasing demand for control of crop pathogens, and a key role of agricultural biotechnology research is to provide safer and more sustainable crop protection strategies. RNA interference (RNAi) is a natural phenomenon which occurs in most eukaryotes whereby small RNA molecules selectively silence the expression of target genes with complementary sequences. Transgenic plants are developed to express double-stranded RNA (dsRNA) and initiate RNAi pathways to control pathogen infection in a technology known as host-induced gene silencing (HIGS). Among many examples of crop pests and pathogens, plant pathogenic fungi have also shown the ability to successfully take up externally applied dsRNA from the environment. Moreover, studies have demonstrated that externally applied dsRNA is able to silence gene expression of target genes which are significant for pathogenicity. These dsRNAs are applied with sprays onto plant hosts in what is known as spray-induced gene silencing (SIGS). SIGS strategies have demonstrated successful disease control through reduced lesion size and fungal biomass in several studies of important crop pathogens, such as *Fusarium graminearum*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum*. New research studies continuously explore the potential of next generation RNA fungicidal developments that utilise natural RNAi pathways in plant-pathogen interactions. This review first discusses the problem faced with GLS disease in maize crop yields and the GLS control strategies available. Thereafter, the mechanism of action of RNAi pathways in plants and fungi and its various applications in biotechnology are discussed. Finally, the application of RNAi as a crop protection strategy to control fungal foliar diseases is addressed.

Keywords: *Cercospora zeina*; dsRNA; GLS; maize; RNAi; SIGS

1.1 Introduction

Maize (*Zea mays* L.) is one of the most abundant cereal crops grown in the world and is an important staple food valued for its nutritional qualities in food and feed. Moreover, maize has valued uses in industrial processes and biofuel. Over 1,200 million metric tonnes of maize were produced globally in 2021/2022 and over 16,3 million metric tonnes were produced in South Africa (USDA, 2022). Naturally occurring diseases of maize threaten the production quantity and quality of this important crop and hence, the global food security. The most common pathogens associated with maize leaf diseases which contribute to yield losses in southern Africa are grey leaf spot (GLS) disease, northern corn leaf blight, common rust, and Phaeosphaeria leaf spot (Nsibo, 2019, Berger et al., 2020, Craze et al., 2022). The GLS and northern corn leaf blight fungal pathogens are members of the class Dothideomycetes (Schoch et al., 2020). The *Cercospora* genus includes various related species that cause the foliar disease collectively classified as GLS disease. GLS is one of the most significant maize diseases in sub-Saharan Africa and the United States of America (USA), with reported grain yield losses of up to 60% in susceptible maize hybrids in South Africa (Ward et al., 1999).

1.2 Grey leaf spot disease

1.2.1 *Cercospora* species are the causal agent of GLS

Grey leaf spot (GLS) is an important disease of maize and the causal agent for this disease in southern Africa is the fungal foliar pathogen *Cercospora zeina* Crous & Braun (Crous et al., 2006, Meisel et al., 2009). The first official report of GLS in South Africa occurred in 1988 in the KwaZulu-Natal province and was identified as *Cercospora zeae-maydis* (Gevers and Lake, 1994). *C. zeae-maydis* Tehon &

Daniels was first described in southern Illinois, USA (Tehon and Daniels, 1925) and has since become a widespread disease of global importance with epidemics appearing in various areas such as the USA, South America, Asia, and Africa (Holliday, 1995, Ward et al., 1999, Dhami et al., 2015, Nega et al., 2016). By the 1992 growing season, GLS reached epidemic levels within South Africa. GLS spread to neighbouring southern African countries such as Zimbabwe, Zambia, Uganda and Kenya by the year 1995 (Ward, 1996, Ward et al., 1999).

A study on the genetic diversity within *C. zeae-maydis* from USA isolates reported that there are two genetically distinct sibling species present which are morphologically similar and have been taxonomically classified as the same species. These species were then classified as groups I and II of *C. zeae-maydis* (Wang et al., 1998). Studies on the genetic similarities between African and USA isolates of *C. zeae-maydis* indicated that African isolates from South Africa, Uganda, Zimbabwe, and Zambia were equivalent to group II, which was found in eastern USA isolates (Dunkle and Levy, 2000). A study which characterised South African *Cercospora* species in terms of genetic differences and morphology, resolved the taxonomic uncertainty and classified *C. zeae-maydis* group II as its own species, namely *Cercospora zeina* Crous & Braun (Crous et al., 2006). The characteristic symptoms of GLS disease includes rectangular, tan lesions that run parallel along the leaf vein that turn grey and necrotic as they mature (Ward et al., 1994) (Fig. 1.1).

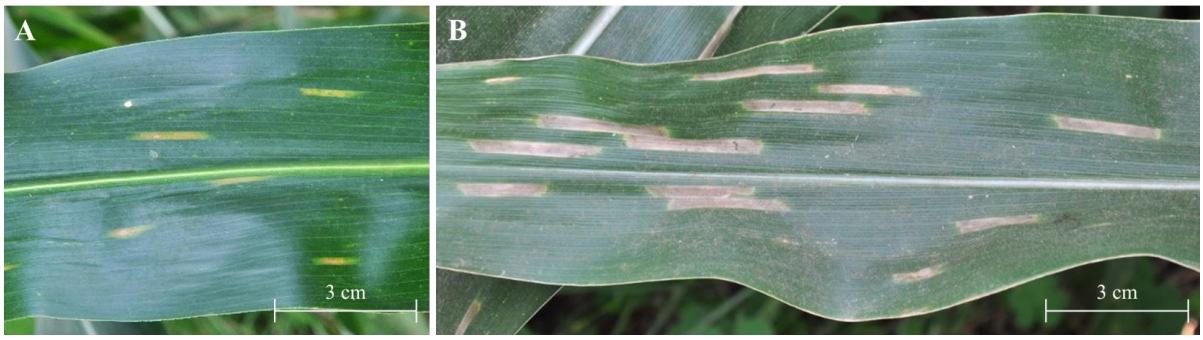

Figure 1.1: Maize fields affected by GLS disease. Foliar GLS symptoms develop from tan, rectangular lesions which run parallel to the leaf vein (A) to grey and necrotic lesions as they mature (B). Diseased maize fields were photographed in Creighton, South Africa (Berger and Nsibo, 2017).

1.2.2 *C. zeina* disease management

The conventional method of controlling GLS disease is to apply chemical fungicides to maize crops and tillage practices are recommended to reduce inoculum in the soil (Latterell and Rossi, 1983, Ward et al., 1999). However, crop rotations and tillage practices are not very effective in areas where GLS is well established, due to neighbouring infected areas spreading inoculum through movement of water in the ground, rain splash, and wind. In seasons with weather conditions that are favourable for GLS, there is no significant difference observed in the infection time of the fungus between maize fields in Cedara (KwaZulu-Natal, South Africa) which practiced tillage and those which did not (Ward et al., 1994). Many studies have been conducted on the response of GLS to commercial fungicides. Research in South Africa has shown that fungicides can provide effective protection from GLS infection for up to 32 days, on the condition that fungicides were applied to leaves with disease levels that were less than 2% of the infected leaf areas (Ward et al., 1996). Maize yield responses to fungicidal treatments depended on the growth stage upon first fungicidal applications, how severe GLS disease infection was at the time of application, and control of disease up until physiological maturity (Ward et al., 1997b). Conventional fungicides that are used to control GLS include demethylase inhibitors (DMI) such as triazole and strobilurin fungicides, and succinate dehydrogenase inhibitors (Ward et al., 1996, Ward et al., 1997a,

Ward et al., 1997b) (Table 1.1). Artea® (Syngenta), Acanto® Plus (DuPont), CUSTODIA®, DIVINO®, MIRADOR® (ADAMA), Nativo®, and Zantara® (Bayer) are all examples of commercial fungicide formulations that fall under the latter categories, and which are available to control GLS in South Africa (Table 1.1).

Table 1.1: Summary of fungicides that are used to control GLS in South Africa.

Fungicide	Manufacturer	Active ingredient(s)	Mode of action	Reference
Artea®	Syngenta AG, Basel, Switzerland	Propiconazole (triazole) and cyproconazole (triazole)	Demethylase inhibitor	(Syngenta, 2022)
Acanto® Plus	DuPont de Nemours, Wilmington, Delaware, USA	Picoxystrobin (strobilurin), cyproconazole (triazole)	Demethylase inhibitor	(Corteva Agriscience, 2019)
CUSTODIA®	ADAMA, Tel Aviv, Israel	Azoxystrobin (strobilurin), tebuconazole (triazole)	Demethylase inhibitor	(ADAMA, 2021a)
DIVINO®	ADAMA, Tel Aviv, Israel	Difenoconazole (triazole)	Demethylase inhibitor	(ADAMA, 2022)
MIRADOR®	ADAMA, Tel Aviv, Israel	Azoxystrobin (strobilurin)	Demethylase inhibitor	(ADAMA, 2021b)
Nativo®	Bayer, Leverkusen, Germany	Trifloxystrobin (strobilurin) and tebuconazole (triazole)	Demethylase inhibitor	(Bayer Crop Science, 2021)
Zantara®	Bayer, Leverkusen, Germany	Bixafen (pyrazolecarboxamide) and tebuconazole (triazole)	Succinate dehydrogenase inhibitors and demethylase inhibitors	(Bayer Crop Science, 2021)

Resistant maize hybrids offer better protection against GLS than crop rotation or tillage practices, however it is generally found that more resistance in hybrids leads to less yield potential (Ward et al., 1994, Gorman et al., 1997, Ward and Nowell, 1998). Many studies in maize crop breeding strategies have been conducted to identify quantitative trait loci (QTL) associated with GLS resistance in the maize genome (Thompson et al., 1987, Gordon et al., 2006, Balint-Kurti et al., 2008, Wisser et al., 2011, Zhang et al., 2012, Mammadov et al., 2015). QTL studies conducted on field conditions in South Africa have found major genes involved in GLS resistance, such as *GLS1*, which can contribute towards maize breeding programmes for crop protection purposes (Gevers and Lake, 1994, Gordon et al., 2004, Berger et al., 2014, Welgemoed et al., 2020). Moderately resistant maize hybrids are often used in conjunction with foliar fungicidal treatments to achieve highest yields in commercial farming (Mallowa et al., 2015). Moderately resistant commercial white or yellow maize hybrids available in southern Africa include the DKC73 varieties (Bayer, 2021), PAN 6Q-865BR (Pannar Seed, 2015), Pioneer P1975 (Pioneer, 2022), and Agricol IMP 51-22BR (Agricol, 2022).

At present, there is no single solution to provide complete crop protection against GLS that comes without unwanted consequences of increased labour, costs, administration of harmful chemical pesticides, or reduced yield potential due to maize hybrid selection. The need for an efficient, environmentally friendly, and cost-effective crop protection strategy against GLS in maize increases with the challenges faced in global food security and trade.

2.1 RNA interference

2.1.1 Introduction

In eukaryotes, small RNAs (sRNA) or small interfering RNAs (siRNA) are short regulatory RNAs which can silence genes with messenger RNA (mRNA) sequences that are complementary to the sRNA sequences (Fig. 1.2). These sRNAs are 20 - 26 nucleotides (nt) in length and are created through the action of a Dicer or Dicer-like (DCL) enzymes with RNA helicase and RNase III domain activity, acting on long double-stranded RNA (dsRNA) (Bernstein et al., 2001). These long dsRNA typically originate from RNA-directed RNA polymerase (RDRP) acting on single-stranded RNAs (ssRNAs), or from ssRNAs folding into a secondary hairpin structure (Baulcombe, 2004). siRNAs associate with Argonaute (AGO) proteins to form an RNA-induced silencing complex (RISC), which binds to its target complementary mRNA sequences (Hammond et al., 2000) (Fig. 1.2). RISC has endonuclease properties and causes cleavage or translational inhibition of target sequences (Vaucheret, 2006). Gene silencing mediated by these siRNAs is referred to as RNA silencing or RNA interference (RNAi) and perform various functions in organisms (Fig. 1.2).

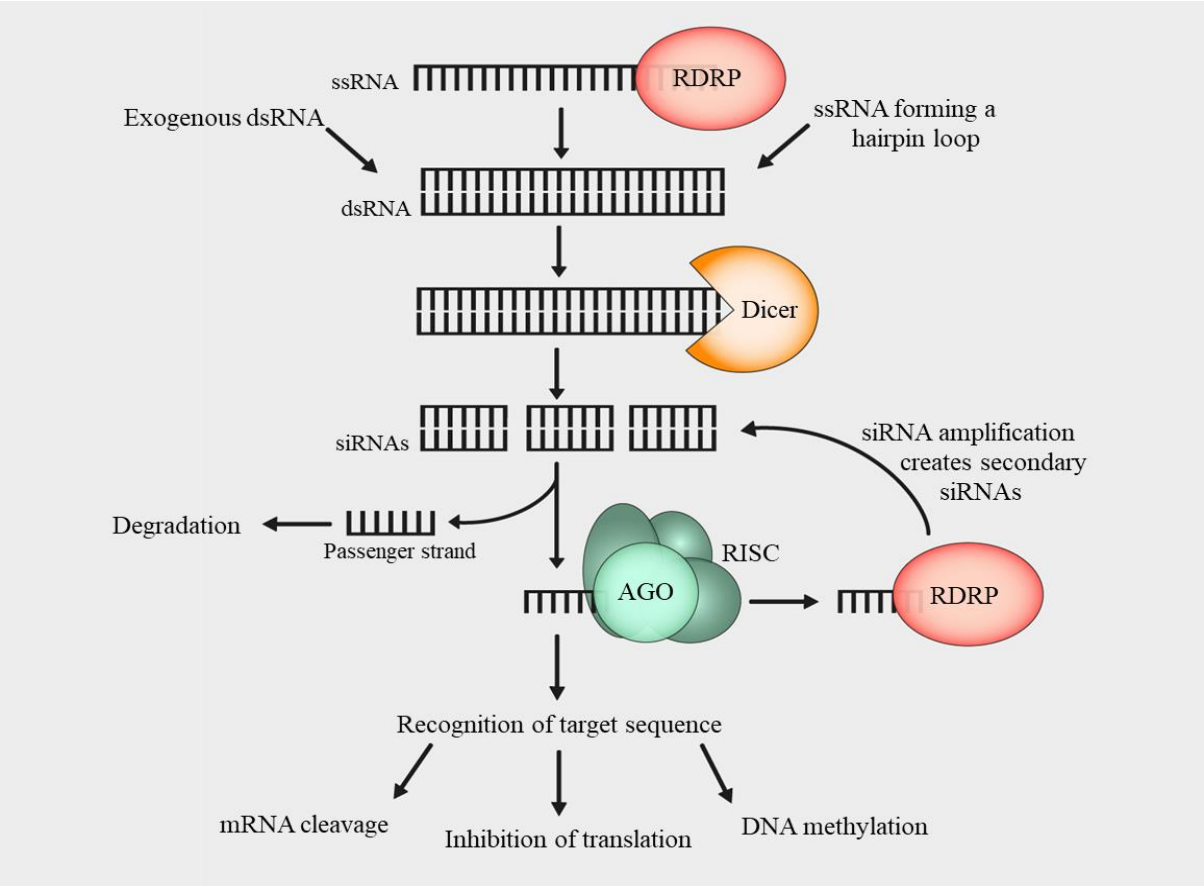

Figure 1.2: Summary of the major enzymes and components involved in RNAi pathways. In eukaryotes, dsRNA originates either from exogenous sources, an RNA-dependant RNA polymerase (RDRP) acting on ssRNA, or secondary RNA structures which allows the ssRNA to fold onto itself and form a hairpin loop. Dicer cleaves the long dsRNA and produces siRNAs. An Argonaute (AGO) associates with a single strand of the siRNA and recruits other enzymes to form an RNA-induced silencing complex (RISC). The remaining passenger strand from the siRNA is degraded. RISC facilitates the recognition of the target sequence. Depending on the nature of the target sequence, either mRNA cleavage, translational inhibition, or DNA methylation occurs. RDRP can associate with siRNAs to create more siRNAs and thus amplify the RNAi signal. Figure designed from several sources.

2.1.2 RNAi in plants

A ground-breaking study in which overexpression of anthocyanin genes was attempted in pigmented petunia petals, led to the discovery of transgene-induced gene silencing in plants. In this study, overexpression of an introduced chalcone synthase gene caused reversible suppression of the gene's expression *in planta* and resulted in inhibition of anthocyanin pigmentation (Napoli et al., 1990, Van der Krol et al., 1990). The gene silencing acted on both the transgene and homologous endogenous genes, and occurred on a post-transcriptional level caused by the production of aberrant RNA (Metzlaff et al., 1997). This study is regarded as one of the first discoveries of RNAi (Vaucheret et al., 1998, Eamens et al., 2008).

2.1.2.1 Dicer-like genes in plants

Studies in *Arabidopsis thaliana* created a mutant line with a knockout of the *carpel factory* (*CAF*) gene, which created a phenotype of abnormal morphology, such as unregulated cell division in floral meristems. When the function of the *CAF* gene was investigated, the resulting CAF protein was found to contain an RNA helicase and RNase domain; an N-terminal DexH/DEAD-box RNA helicase-type domain and a C-terminal RNaseIII-like domain. They hypothesized that CAF plays a role in RNA processing events (Jacobsen et al., 1999). Further studies elucidated the role of CAF to be one of the Dicer-like (DCL) proteins, namely DCL1 (Schauer et al., 2002). *Arabidopsis* is known to have four *DCL* genes (*DCL1* through *DCL4*) and the function of *DCL1* has been characterized to be involved in the formation of siRNAs and micro RNAs (miRNAs) (Xie et al., 2004, Margis et al., 2006). Thus, the *Arabidopsis* mutant line with a knockout of the *CAF* gene (now known to be *DCL1*) was incapable of processing sRNAs from endogenous dsRNAs, which caused the observed phenotype of defective plant organs (Park et al., 2002). In addition to the four *DCL* genes identified in *Arabidopsis*, eight *DCL* genes in rice, and five *DCL* genes in maize have been identified (Qian et al., 2011).

2.1.2.2 Argonaute in plants

A mutant screen performed in *Arabidopsis* revealed that plants with the *AGO1* gene knockout for Argonaute 1 (AGO1) protein resulted in defective morphological characteristics such as pointed cotyledons, narrow rosette leaves, and abnormal inflorescences (Bohmert et al., 1998). The *AGO1* gene was found to be a member of gene orthologs that include *RNA interference-deficient* gene, *RDE-1*, present in the model organism, the roundworm nematode *Caenorhabditis elegans*. RNAi was greatly impaired in *RDE-1* knockout mutants of *C. elegans* (Tabara et al., 1999). There are 10 members of the AGO family in *Arabidopsis* and their function has been revealed to be RNA-binding proteins which recruit siRNAs and miRNAs for RNase activity. *AGO1* selectively associates with siRNAs and miRNAs, and forms RISC, which possesses an RNase activity that cleaves target complementary mRNA (Hammond et al., 2000, Baumberger and Baulcombe, 2005). A total of 19 *AGO* genes have also been identified in rice, and 18 *AGO* genes have been identified in maize (Qian et al., 2011).

2.1.2.3 Functions of RNAi pathways in plants

Three primary functions have been identified in the natural RNAi pathway in plants: i) defence against invading viruses, ii) miRNA regulation during abiotic stress and development, iii) and siRNA-directed DNA methylation which protects the genome from transposons and plays a role in the regulation of heterochromatin formation (Baulcombe, 2004) (Fig. 1.3).

2.1.2.4 Antiviral defence

Cytoplasmic siRNA silencing is a defence mechanism deployed by plant immune responses against invading viruses, where viral dsRNA structures are recognised. dsRNA is produced by many viruses as replication intermediate RNA viral structures. Alternatively, dsRNA is produced from viral ssRNA which has either folded onto itself or which has been made into dsRNA by the action of the plant RNA-dependent RNA polymerase (Ratcliff et al., 1997, Waterhouse et al., 1998, Baulcombe, 2015). The viral dsRNA is processed by the host RNAi pathway to produce siRNAs (Fig. 1.3). Dicer-like 2 (DCL2) is

known to be involved in the creation of viral siRNAs (Xie et al., 2004). The siRNAs act as guides for AGO1 to silence the reverse complement of viral genes (Fig. 1.3).

In response to the antiviral defence system in plants, viruses encode viral suppressors of RNA silencing (VSS) which fundamentally act as virulence effectors (Csorba et al., 2015). One of the most well-known examples of VSS, is the Tombusvirus protein, P19, which binds siRNAs and suppresses the RNA silencing pathway (Qu and Morris, 2002, Scholthof, 2006). P19 has also been shown to interact with two plant plasma membrane-localized receptor-like kinases, BAM1 and BAM2 (Rosas-Díaz et al., 2020). Studies in *Arabidopsis* propose that BAM1/2 are responsible for the cell-to-cell spread of siRNA and for the distribution of RNA silencing throughout the vascular system (Rosas-Diaz et al., 2018). Thus, P19 is often used in viral expression vectors to silence the antiviral response in transgenic plants and to increase the expression yield of the transgene production in biopharming and studies that use plants transformed by virus-based vectors (Fischer et al., 2004, Mohammadzadeh et al., 2016, Rössner et al., 2022).

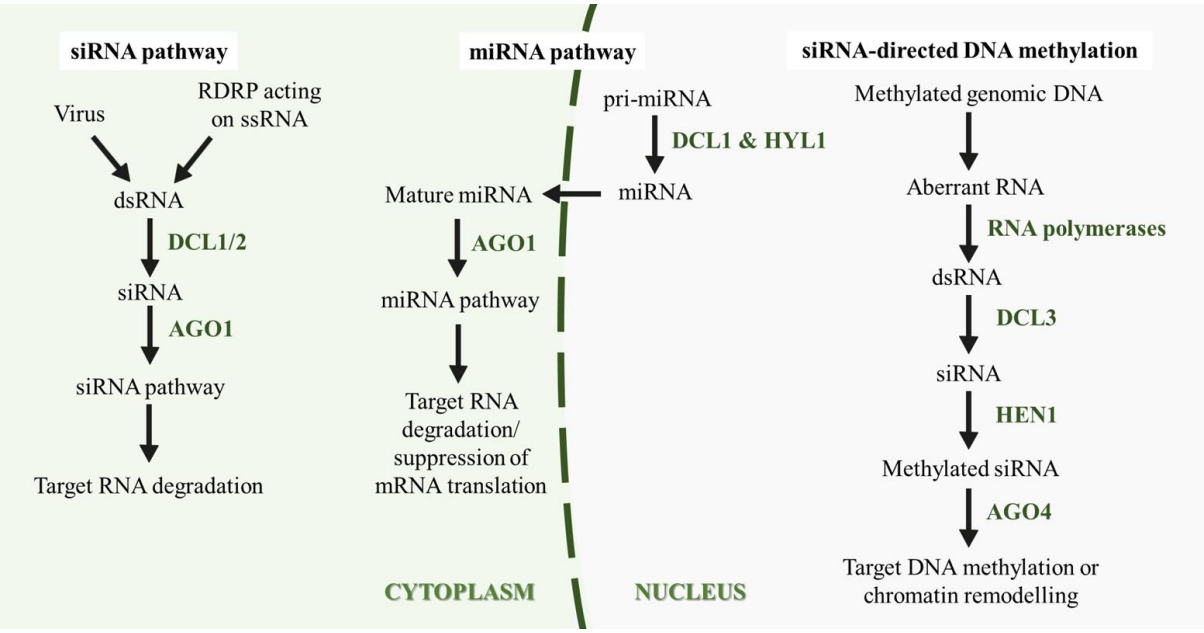

Figure 1.3: Summary of three major RNAi pathways in plants. The siRNA pathway involves dsRNA that originates from a viral source or an RNA-dependent RNA polymerase (RDRP) acting on ssRNA. This dsRNA is processed by Dicer-like proteins (DCL1 or DCL2) and produces siRNAs which associate with the Argonaute, AGO1, to activate the siRNA pathway that is used in viral defence. In the miRNA pathway, primary miRNAs (pri-miRNA) are processed in the nucleus by DCL1 and Hyponastic leaves 1 (HYL1) to produce mature miRNAs which are transported to the cytoplasm. The miRNAs associate with AGO1 and the miRNA pathway is initiated. The miRNA pathway degrades target RNA sequences or performs posttranscriptional silencing through suppression of mRNA translation in the regulation of plant development and physiology. In siRNA-directed DNA methylation, methylated DNA sequences in the genome can produce aberrant RNA which is produced into dsRNA by the action of either RNA polymerase II, RDR2, or RNA polymerase PolIVa. This dsRNA is processed by DCL3 and the resulting siRNA is methylated by Hua Enhancer 1 (HEN1). The resulting siRNAs associate with AGO4 and conduct siRNA-directed DNA methylation, a process which involves a chromatin-remodelling protein, Defective in RNA-directed DNA methylation 1, RNA polymerase PolIVb, and Domains rearranged methylase 2. Figure designed from several sources.

2.1.2.5 Regulation of development and physiology

The second known RNAi pathway is RNA silencing of endogenous mRNA by miRNAs, which are also derived by Dicer-like protein cleavage of miRNA gene transcripts (Lee and Ambros, 2001) (Fig. 1.3). sRNAs are classified as either siRNAs, which are processed from long dsRNAs, or miRNAs, which are produced by a long ssRNA which folds back unto itself to produce a dsRNA with a stem loop. siRNAs and miRNAs are typically 21-24 nt in length. The miRNA precursors are derived from RNA structures that contain inverted repeat sequences. These precursors, named primary miRNAs (pri-miRNAs), are processed in the nucleus by DCL1 and the dsRNA-binding protein Hyponastic leaves 1 (HYL1) to produce 21 nt long miRNAs (Dong et al., 2008) (Fig. 1.3). The mature miRNAs are transported to the cytoplasm where they associate with AGO1 and the zinc-finger protein Serrate (Dong et al., 2008, Baumberger and Baulcombe, 2005). These miRNAs target mRNAs with complementary sequences resulting in translational inhibition or cleavage. This functions to modulate gene expression of endogenous genes and regulates various key developmental and abiotic stress responses (Aukerman and Sakai, 2003, Sunkar et al., 2007). Many miRNAs are transcribed from *miRNA* genes and are involved in the regulation of developmental processes such as leaf morphogenesis (Palatnik et al., 2003, Axtell et al., 2007). In addition, miRNAs have been demonstrated to regulate environmental responses. The model legume *Medicago truncatula* expresses a miRNA, miR171H, which controls the transcript levels of a transcription factor involved in root nodule formation. This gene silencing prevents the over-colonisation of roots by mycorrhizal fungi (Lauressergues et al., 2012). Rice plants possess a miRNA mechanism which regulates an endogenous gene, *OSNRAMP6*, to confer resistance to the blast fungus *Magnaporthe oryzae* (Campo et al., 2013, Li et al., 2014).

The *trans*-acting siRNA (tasiRNA) pathway is a phenomenon whereby miRNAs bind to their target transcripts and instead of causing gene silencing, these transcripts are cleaved and subsequently used as templates for RNA-directed RNA polymerase 6 (RDR6) to create dsRNA. Thus, the tasiRNA control expression of their own mRNA transcripts by means of post-transcriptional RNA silencing. An example of the tasiRNA pathway is found in the regulation of auxin response factors in *Arabidopsis* (Howell et al., 2007). Another example of endogenous sRNAs regulating the expression of homologous mRNAs is found in the natural-antisense siRNA (natsiRNA) pathway. These endogenous *cis* genes create natural antisense transcripts that allow for sequence-specific targeting of complementary *cis*-sense gene transcripts (Eamens et al., 2008). The biogenesis of natsiRNA start with transcription of different DNA strands which contain sequence complementarity at their 3'- or 5'-ends and subsequently produce dsRNA (Bouchard et al., 2015). The dsRNA is cleaved by DCL2 and generates 24 nt natsiRNA segments which target *cis*-antisense gene transcripts for cleavage (Borsani et al., 2005). The cleaved products become the substrate for RDR6 and Suppressor of gene silencing 3 (SGS3) to produce dsRNA molecules that are cleaved by DCL1 into 21 nt natsiRNA segments. These smaller natsiRNA products act as RNA silencing agents for homologous mRNA on a post-transcriptional level. The initiation of this pathway has been observed in response to abiotic factors in *Arabidopsis* such as salt stress and thermotolerance (Borsani et al., 2005, Li et al., 2020).

2.1.2.6 Maintenance of genome integrity

A third known RNAi pathway is associated with transcriptional gene silencing through the regulation of histone modifications and heterochromatin formation by means of RNA-directed DNA methylation of target nucleotide sequences (Wassenegger et al., 1994, Volpe et al., 2002, Zilberman et al., 2003, Matzke et al., 2007) (Fig. 1.3). DNA methylation is an epigenetic modification that regulates gene expression and repetitive sequences in the genome, such as transposons. Repeat sequences in the *Arabidopsis* genome have been found to be the source of many repeat-associated siRNAs, which direct DNA methylation and thus regulate repeat DNA sequences in the genome (Chan et al., 2005). Aberrant RNA is transcribed from methylated DNA and processed into dsRNA by either RNA polymerase II, RDR2, or RNA polymerase PolIVa (Wassenegger and Pélissier, 1998, Eamens et al., 2008) (Fig. 1.3). Dicer-like 3 (DCL3) processes this dsRNA into 24 nt siRNAs which are then methylated by the sRNA-specific methyltransferase Hua Enhancer 1 (HEN1) (Chen et al., 2002). Argonaute 4 (AGO4) directs

the RNA-directed DNA methylation of cognate target DNA sequences, a process which involves a chromatin-remodelling protein, Defective in RNA-directed DNA methylation 1, RNA polymerase PolIVb, and Domains rearranged methylase 2 (Kanno et al., 2005). Methylated DNA is then maintained by canonical DNA methyltransferases.

It has been shown that AGO4 is part of a silencing machinery that produces 25 nt siRNA, which is responsible for gene silencing at chromatin level, specifically Histone 3 lysine-9 (H3K9) methylation (Zilberman et al., 2003). The latter pathway likely functions to protect the genome from transposons (Lippman et al., 2004) and this phenomenon has also been identified in filamentous fungi with the function to protect against retrotransposons and the control of heterochromatin formation (Volpe et al., 2002, Nolan et al., 2005).

2.1.3 RNAi in fungi

The key components of RNAi, which are believed to be conserved across most eukaryotes, are Argonautes, Dicers, and RNA-dependent RNA polymerases (RDRPs) (Fig. 1.2 and Table 1.2). However, there are certain fungal taxa which do not possess all these key components, for example budding yeasts (Nakayashiki et al., 2006). In fungi, RNAi mechanisms have been studied in the model organisms, *Neurospora crassa* and *Schizosaccharomyces pombe*. In filamentous fungi, such as *N. crassa*, RNAi occurs post-transcriptionally whereas in yeasts, such as *S. pombe*, RNAi occurs on the transcriptional gene silencing level with the control of heterochromatin formation (Volpe et al., 2002, Moazed, 2009). Some of the major homologous proteins involved in RNAi pathways in plants and fungi have been summarised in Table 1.2.

2.1.3.1 Quelling in Neurospora crassa

Early studies of RNAi in fungi were conducted on *N. crassa*, where mechanisms such as quelling and meiotic silencing by unpaired DNA have been observed. Quelling is a term used to describe a phenomenon in *N. crassa* which is similar to the co-suppression observed in 1990 by Napoli *et al.* in petunias (Romano and Macino, 1992). In this study, wild-type strains with a bright orange phenotype were transformed with exogenous plasmids which contained gene sequences for carotenoid pigment genes. The resulting transformants were of an albino phenotype, ranging from white to dark yellow. They observed that the exogenous sequences introduced through transformation of the fungi were inactivating the expression of the corresponding endogenous genes and named this phenomenon quelling. This study also demonstrated that over a prolonged culturing period, around 20 – 27% of the transformants with albino phenotypes reverted to wild-type or intermediate phenotypes. Thus, the transcriptional inactivation of the pigment genes was unstable and some of the growing cells reversed the quelling phenomenon to revert to the wild-type phenotype. Moreover, they demonstrated that quelling was unidirectional and that once reversed phenotypes were observed, further quelling did not naturally take place during prolonged culturing. They confirmed that quelling takes place on a transcriptional level and that phenotypes of various levels of quelling and reversal showed corresponding levels of mRNA transcripts for the target genes. DNA methylation analysis of the target genes in the transformants suggested that DNA methylation could be involved in quelling (Romano and Macino, 1992).

Further support for the transgene-induced gene silencing observed in quelling was found in *N. crassa.* Studies in *N. crassa* created *quelling-defective* (*qde*) mutants which identified three loci, *QDE-1*, *QDE-2*, and *QDE-3* (Cogoni et al., 1996, Cogoni and Macino, 1997) (Table 1.2). Recessive *qde* mutants were impaired in gene silencing and accumulated RNA transcripts of the transgene. These studies also confirmed that the presence of transgenic sense RNA is required for the gene silencing observed in *N. crassa*. Further studies on *qde* mutants revealed that the *QDE* genes involved in gene silencing, are genes which encode for RNAi machinery such as the RDRP protein (from the *QDE-1* gene), an Argonaute protein (from *QDE-2*), and a RecQ DNA helicase (from *QDE-3*) (Cogoni and Macino, 1999a, Cogoni and Macino, 1999b, Catalanotto et al., 2000) (Table 1.2). Furthermore, these studies

enabled the characterisation of two *dicer-like* genes in *N. crassa* (*DCL1* and *DCL2*) and that double *dcl* mutants were incapable of quelling, while single mutants showed that the two *DCL* genes are redundant (Catalanotto et al., 2004). In wild-type cells, the dsRNA is processed by the dicers, DCL1 and DCL2, to produce siRNAs of 20-25 nt. The siRNAs are loaded onto RISC which is formed by the *N. crassa* Argonaute protein (QDE-2) and an associated exonuclease, QDE-2 interacting protein (QIP) (Maiti et al., 2007).

2.1.3.2 DNA damage repair in Neurospora crassa

Quelling is also induced by DNA damage. Studies in *N. crassa* have shown that DNA damage induces the production of sRNAs of 20-21 nt in length which in turn activate the expression of the Argonaute protein QDE-2. These sRNAs have been called QDE-2-associated sRNAs (qiRNAs) (Lee et al., 2009). These qiRNAs have a strong bias for 5' uracil and are derived from endogenous highly repetitive sequences of ribosomal DNA loci. These qiRNAs may act as a DNA damage checkpoint by preventing translation. The proposed DNA damage response RNAi pathway is initiated by double-stranded breaks which have been introduced by DNA damage or tandem repeats. These double-stranded breaks then cause aberrant replication. The siRNA pathway is triggered, and homologous recombination of repeat sequences takes place (Zhang et al., 2013). This forms recombination intermediates which are recognised by the RDRP protein QDE-1 and the helicase QDE-3 to produce dsRNA. The dsRNAs are processed by Dicer and QDE-2 to perform post-transcriptional gene silencing of homologous RNAs (Yang et al., 2015, Torres-Martínez and Ruiz-Vázquez, 2017) (Table 1.2).

2.1.3.3 Meiotic silencing by unpaired DNA (MSUD) in Neurospora crassa

Another post-transcriptional gene silencing mechanism, called meiotic silencing by unpaired DNA (MSUD), occurs in *N. crassa* during sexual development (Shiu et al., 2001). If one purpose of quelling could be thought of as a mechanism to silence DNA sequences which have more than one copy in the haploid phase, MSUD can be considered a mechanism to silence DNA sequences which do not have two copies, or an odd number of copies, in die diploid zygote phase. During the prophase of meiosis I, MSUD silences unpaired DNA sequences. In addition, any unpaired DNA sequences can cause gene silencing of homologous DNA segments, even if the silenced target sequences are themselves paired. This silencing mechanism could provide defence against transposable elements that move during meiosis (Shiu et al., 2001).

This study also illustrated that a knockout mutant for the *suppressor of ascus dominance 1* (*SAD-1*) gene does not execute the MSUD pathway and that the *SAD-1*$^{+}$ wild-type allele encodes for an RDRP and is a paralog of QDE-1 (Shiu et al., 2001) (Table 1.2). A paralog of the QDE-2 Argonaute protein, Suppressor of meiotic silencing 2 (SMS-2), was found to be required in the MSUD pathway (Lee et al., 2003). Suppressor of ascus dominance 1 (SAD-1) is located by, and interacts with, SAD-2, and creates dsRNA together with an RNA/DNA helicase (SAD-3) (Table 1.2). The dsRNA is cleaved by DCL1. The resulting siRNA associate with SMS-2 and the exonuclease, QDE-2 interacting protein (QIP), to silence homologous genes (Billmyre et al., 2013). Quelling and the MSUD pathways have QIP and DCL1 in common (Table 1.2), suggesting that these two pathways might have shared ancestry (Chang et al., 2012). The most recent proteins identified to play a key role in MSUD are the Suppressor of ascus dominance proteins, SAD-4 and SAD-5. SAD-4 localises to the perinuclear region and SAD-5 is found in the nucleus (Hammond et al., 2013).

Table 1.2: Summary of some of the major proteins and genes involved in RNAi pathways in plants and fungi. Proteins involved in RNAi pathways were selected from studies in *A. thaliana* to represent plant protein homologs, and proteins from studies in *N. crassa* were selected to represent fungal protein homologs.

Protein	Homologous protein and its function in *A. thaliana*	Homologous protein and its function in *N. crassa*
RNA-directed RNA polymerase	**RDR6** acts on ssRNA to produce dsRNA (Peragine et al., 2004)	**QDE-1** acts on ssRNA to produce dsRNA (Cogoni and Macino, 1999a)
	RDR2 is involved in maintenance of genome integrity (Chan et al., 2004)	**SAD-1** is a paralog of QDE-1 and is involved in the MSUD pathway (Shiu et al., 2001)
Dicers	**DCL1** cleaves dsRNA to produce siRNAs (Schauer et al., 2002), processes pri-miRNAs (Dong et al., 2008), and is involved in the biogenesis of natsiRNA (Borsani et al., 2005)	**DCL1** and **DCL2** cleaves dsRNA to produce siRNAs (Catalanotto et al., 2004). These Dicers process dsRNAs in a DNA damage repair pathway mediated by quelling (Torres-Martínez and Ruiz-Vázquez, 2017), and cleave pri-milRNA to produce pre-milRNA (Lee et al., 2010)
	DCL2 processes viral dsRNA into siRNAs (Xie et al., 2004) and is involved in the biogenesis of natsiRNA (Borsani et al., 2005)	
	DCL3 is involved in chromatin modification and RNA-directed DNA methylation (Chen et al., 2002)	**DCL1** is involved in the MSUD pathway (Billmyre et al., 2013)
Argonaute	**AGO1** binds siRNAs and forms part of RISC (Hammond et al., 2000), and is involved in the processing of pri-miRNAs (Dong et al., 2008)	**QDE-2** recruits QIP and loads siRNA onto RISC (Maiti et al., 2007), is involved in DNA damage repair (Catalanotto et al., 2000), and processes pre-milRNA (Lee et al., 2010)
	AGO4 is involved in RNA-directed DNA methylation (Zilberman et al., 2003)	**SMS-2** is a paralog of QDE-2, it associates with QIP, and is involved in the MSUD pathway (Lee et al., 2003)
Helicase	**DCL1** has an RNA helicase domain (Jacobsen et al., 1999)	**QDE-3** is a RecQ DNA helicase involved in DNA damage repair (Cogoni and Macino, 1999b)
		SAD-3 is an RNA/DNA helicase involved in the MSUD pathway (Billmyre et al., 2013)

2.1.3.4 Antiviral defence in fungi

Many eukaryotes evolved RNAi machinery as a mechanism for the defence against viruses, as is the case in fungi. The first evidence of RNAi utilised as antiviral defence was reported in the chestnut blight fungus, *Cryphonectria parasitica*. This fungus is known to host the replication of RNA mycoviruses. Experiments which disrupted the *dicer-like* gene, *DCL2*, resulted in increased Cryphonectria hypovirus 1 (CHV1) mycovirus infection (Segers et al., 2007). Knock-out mutants for RNA silencing genes in *Colletotrichum higginsianum* indicated that *dcl1* and *ago1* mutants suppressed a dsRNA mycovirus, named *Colletotrichum higginsianum non-segmented dsRNA virus 1* (ChNRV1) (Campo et al., 2016). This study also reported reduced conidiation and an increase in defects observed in conidia morphology caused by the ChNRV1 virus in the *dcl1* and *ago1* knockout *C. higginsianum* mutants. These defects included shorter and wider conidia compared to the wild-type control strain. Interestingly, mycoviruses can be both the targets and the suppressors of fungal RNA silencing. Studies have reported this in the interactions between *Aspergillus nidulans*, *Rosellinia necatrix*, and their associated mycoviruses (Hammond et al., 2008, Yaegashi et al., 2013).

2.1.3.5 Environmental signal responses in fungi

Endogenous sRNAs (esRNAs) are produced from and regulate endogenous gene sequences. There are various classes of esRNAs, one of which is microRNA-like small RNAs (milRNAs). milRNAs were first described in *N. crassa* as approximately 25 nt long sRNAs, which are produced from stem-loop RNA precursors that are transcribed from endogenous intergenic regions (Lee et al., 2010). While the milRNA biogenesis pathway is similar to those of canonical miRNAs, it is not identical. In the *Neurospora* genome the *MILR-1* locus produces the most abundant milRNAs. While the majority of miRNA biogenesis relies on RNA Pol II, *N. crassa* sRNA analysis indicated that RNA Pol II and III transcribe various milRNAs to pri-milRNA (Yang et al., 2013). The pri-milRNA transcript contains a hairpin structure which is then cleaved by Dicer to produce a smaller dsRNA product, called a pre-milRNA. The Argonaute protein QDE-2 interacts with pre-milRNA and recruits the exonuclease QIP to process the pre-milRNA and to produce mature milRNAs (Table 1.2). The milRNAs mediate gene silencing through translational repression (Lee et al., 2010).

MicroRNA-like small RNAs have since been identified in other filamentous fungi, including plant pathogens such as *Fusarium graminearum, F. oxysporum, Sclerotinia sclerotiorum, Rhizoctonia solani* and the maize leaf spot pathogen *Curvularia lunata* (Zhou et al., 2012, Chen et al., 2014, Chen et al., 2015b, Lin et al., 2016, Liu et al., 2016). The functions of milRNAs are poorly understood but literature suggests that these sRNAs are involved in fungal responses to environmental signals and in pathogenesis through participation in germination and mycelial growth processes (Torres-Martínez and Ruiz-Vázquez, 2017). Similar findings were reported in *Verticillium dahliae*, a fungal pathogen causing wilt disease in various crops. *V. dahliae* uses a milRNA, VdmilR1, to target endogenous virulence genes which regulates fungal pathogenicity during infection (Jin et al., 2019).

Reports of milRNAs show a diverse range of target genes, suggesting that milRNAs evolved as a mechanism to provide for adaptation to various environmental conditions using sRNA regulation (Villalobos-Escobedo et al., 2016). A remarkable example of the involvement of RNAi pathways in adaptation to environmental conditions was observed in *Mucor circinelloides* which was able to develop fungicidal resistance. FK506 exerts antifungal activity by binding to the fungal protein FKBP12. The FK506-FKBP12 complex inhibits calcineurin and as a result blocks hyphal growth and the fungus remains in yeast-phase growth (Liu et al., 1991). *M. circinelloides* uses RNAi to produce epigenetic changes to silence the endogenous *FKBP12* gene, rendering the resulting epimutant to be resistant to the antifungal treatment (Calo et al., 2014). Interestingly, FK506-resistant epimutants reverted to the wild-type when grown in the absence of FK506.

2.1.3.6 Regulation of development and physiology in fungi

MicroRNA-like small RNAs have also been observed to exhibit differential transcription during mycelial growth, spore development, and germination. These findings suggest milRNA involvement in developmental processes, particularly with regards to fungal pathogenesis (Lin et al., 2015, Liu et al., 2016).

Endogenous gene silencing by esRNAs has been characterized in the basal fungus *Mucor circinelloides*. The RNA polymerase RDRP1 transcribes the antisense strand to produce dsRNA, which is then processed by DCL2 to produce siRNAs of either 21 or 25 nt in length (Torres-Martínez and Ruiz-Vázquez, 2016) (Table 1.2). Studies in *M. circinelloides* vegetative growth showed that AGO1 binds to the siRNAs to silence target transcripts (Cervantes et al., 2013). Residues of processed target mRNAs are used as templates by RDRP2 to generate new dsRNA molecules and thus amplify the gene silencing signal (Calo et al., 2012). A similar amplification signal caused by siRNAs has been observed in the fission yeast *S. pombe* (Simmer et al., 2010).

Studies in *M. circinelloides* have described a different class of esRNAs which are derived from exons of protein-coding sequences and regulate the expression of corresponding mRNAs (Nicolas et al., 2010). The biogenesis of most of these exon-derived regulatory siRNAs (ex-siRNAs) are dependent on the Dicer enzyme. There are four classes (class 1-4) of Dicer-dependent ex-siRNAs and class 2 is the main class. DCL2, RDRP1, and AGO1 enzymes are involved in the biogenesis of ex-siRNAs, except in class 1 which requires RDRP2 instead of RDRP1. Class 3 and 4 are similar in that they require both RDRP1 and RDRP2. However, class 3 requires either DCL1 or DCL2, while class 4 only depends on DCL1. Other features involved in the classification of these ex-siRNAs include size, bias for uracil at the 5'-end, and whether they bind to AGO1 or not (Nicolas et al., 2010, Cervantes et al., 2013, Torres-Martínez and Ruiz-Vázquez, 2016). Gene targets of ex-siRNAs include the direct target genes from which these esRNAs were derived (*cis* regulation) as well as secondary target genes due to a degree of target sequence complementarity with the ex-siRNAs (*trans* regulation). Asexual spore production, accelerated autolysis, and nutrient sensing are examples of developmental processes regulated by RNAi pathways and literature suggests that these are regulated by class 1 ex-siRNAs (Torres-Martínez and Ruiz-Vázquez, 2016).

Studies in *M. circinelloides* described an esRNA pathway, dependent on RDRP but not on Dicer, known as RDRP-dependent degraded RNAs (rdRNAs). The participants in this non-random degradation pathway include RDRP1, RDRP2, and an RNase III-like enzyme, R3B2. These rdRNAs do not show the same strand bias as the canonical ex-siRNAs and are not the typical 20 – 26 nt discrete sizes of Dicer-dependent sRNAs, but instead display no discrete sizes in a range of 16 – 26 nt in length (Trieu et al., 2015). This RNAi pathway has been demonstrated to regulate the expression of conserved genes involved in developmental processes such as the transport and metabolism of co-enzymes, inorganic ions, and secondary metabolites. In addition, regulation of genes involved in intracellular trafficking and secretion was also observed (Trieu et al., 2015).

2.1.3.7 Genome integrity in fungi

Endogenous sRNAs that are Dicer-independent (disiRNAs) are not produced by the known canonical RNAi machinery. Studies in *Neurospora* demonstrated that disiRNAs are produced from genomic regions with no apparent sequence motifs. Instead, these are loci where transcription takes place from opposite directions and produce naturally overlapping sense and antisense transcripts (Lee et al., 2010). disiRNA loci are linked to DNA methylation in what is known as disiRNA loci DNA methylation (DLDM). DLDM is triggered by convergent transcription and accumulates at promoter regions. DLDM is maintained differently from repeat-induced point mutations (RIP), the relatively stable genome protection strategy to silence duplicated DNA sequences during the sexual cycle. In contrast to RIP, DLDM has a highly dynamic nature with an on-off pattern and most of the DLDM regions are not

extensively methylated (Dang et al., 2013). disiRNAs regulate expression of sense target transcripts; they enable DNA methylation, recruitment of enzymes such as an exonuclease (ERI-1), and consequentially recruit histone methyltransferases involved in chromatin modifications (Dang et al., 2016). This pathway has been observed to be involved in the regulation of the circadian clock *frequency* (*FRQ*) gene in *N. crassa* (Kramer et al., 2003, Dang et al., 2013).

In *S. pombe*, it has been demonstrated that centromeric heterochromatin formation is induced by RNAi machinery. Repetitive sequences in centromeres are transcribed by RNA Pol II and produce non-coding RNAs (ncRNAs) which contains a trimethylguanosine cap (Yu et al., 2021). An RNA-directed RNA polymerase, RDP1P, produces dsRNA from the ncRNAs, which are processed by Dicer into siRNAs. The siRNAs associate with proteins which include an Argonaute protein, AGO1P, and form a RNAi-induced transcriptional silencing (RITS) complex. The RITS complex recruits another complex, CLRC, which contains a methyltransferase and promotes the methylation of histone H3 at lysine 9 (to form H3K9me2). H3K9me2 provides a binding site for a heterochromatin protein to produce heterochromatin. The formation of heterochromatin allows for the epigenetic silencing of specific loci in the chromosome (Verdel et al., 2004, Kajitani et al., 2017, Mutazono et al., 2017, Yu et al., 2021).

2.1.3.8 Evolution and loss of RNAi in fungi

Phylogenetic analyses of fungal Argonaute, Dicer, and RDRP-like proteins suggest that RNAi mechanisms are ancient pathways which originated before the divergence of major fungal lineages (Nakayashiki et al., 2006). There is a remarkable diversification of RNA silencing pathways in fungal lineages which supports the idea that RNAi mechanisms evolved to support adaptation and complex responses to environmental conditions. Interestingly, loss of RNAi has been observed in some fungi such as the model yeast, *Saccharomyces cerevisiae*, and the maize smut pathogen, *Ustilago maydis* (Billmyre et al., 2013).

Table 1.2 summarises some of the major proteins involved in RNAi pathways in plants and fungi. These eukaryotes require the action of an RNA-dependent RNA polymerase (RDRP) to create dsRNA structures from ssRNA. The QDE-1 polymerase in fungi has a similar role and is an ortholog of the RDR6 RNA-directed RNA polymerase in plants (Zong et al., 2009). Dicer-like enzymes cleave the dsRNA and are loaded by an Argonaute protein that associate with other compounds to form silencing complexes. In *N. crassa*, Dicer-like 1 and 2 (DCL1 and DCL2) process dsRNA into siRNAs (Catalanotto et al., 2004), whereas DCL1 has a role as one of the major Dicer-like enzyme in plants (Henderson et al., 2006). Fungi recruit additional helicases to assist Dicer-like enzymes in cleaving during certain pathways, whereas the plant DCL1 has inherent helicase activity (Bernstein et al., 2001). The major Argonaute which is involved in the miRNA pathway and binds siRNA to form part of RISC in plants is AGO1 (Baumberger and Baulcombe, 2005), whereas this role is performed by QDE-2 in fungi (Lee et al., 2010).

2.1.4 Cross-kingdom RNAi

Double-stranded RNA-mediated interference has been shown to cross cellular boundaries. This phenomenon was first reported in the model organism, the roundworm *C. elegans* (Fire et al., 1998). sRNAs were also discovered to be mobile within plant cells, plant tissues, and between plant host and associated organisms (Baulcombe, 2015). Within the plant, sRNAs can move from cell to cell through the plasmodesmata and perform gene silencing in remote cells or tissues in what is known as systemic acquired silencing (Voinnet et al., 1998, Brosnan and Voinnet, 2011, Sarkies and Miska, 2014).

Small RNAs can move between a plant host and an associated organism, such as a pathogen, to silence target genes in a phenomenon known as cross-kingdom RNAi (Weiberg et al., 2013, Wang et al., 2016). Recent observations in the *Arabidopsis thaliana – Botrytis cinerea* pathosystem described bidirectional cross-kingdom RNAi where the necrotrophic fungus sends sRNAs to its host to supress the expression of defence-related plant genes and thus allow for fungal colonisation (Wang et al., 2016, Cai et al., 2018a). The fungus produces dsRNA which is processed by fungal Dicer enzymes to produce siRNA.

These siRNA are capable of travelling to the plant host cells, where they associate with plant AGO1 proteins to guide the silencing of target plant genes (Weiberg et al., 2013). There are several examples of cross-kingdom RNAi reports of fungal phytopathogens sending sRNAs to act as effectors in their plant hosts, such as *Puccinia striiformis* f. sp. *tritici* and wheat (Wang et al., 2017), and *Phytophthora infestans* and potato (Hu et al., 2022).

A recent study investigated the potential target genes of miRNAs and siRNAs predicted in *B. cinerea* that infected tomato. The findings indicated that miRNA target genes encoded for proteins that were generally: i) located in the membrane and organelles, ii) had protein binding and catalytic properties, and iii) were related to carbohydrate metabolism and signal transduction (Liu et al., 2022).

In the plant's counter defence response, plants produce sRNAs which silence the expression of virulence genes from invading pathogens (Cai et al., 2018b). Verticillium wilt, caused by the fungal pathogen *V. dahliae*, exhibits cross-kingdom RNA silencing as a pathogenicity mechanism in its host (Wang et al., 2016). In the arms race between host and pathogen, the cotton host exports miRNAs to silence specific *V. dahliae* genes. The two reported *V. dahliae* genes encode for a Ca^{2+}-dependent cysteine protease (CLP-1) and an isotrichodermin C-15 hydroxylase (HIC-15) (Zhang et al., 2016, Zhang et al., 2022).

In the continuing warfare between host and pathogen, it has been found that the oomycetal pathogen *Phytophtora* expresses virulence effector proteins that function as suppressors of RNAi pathways in plants. The *Phytophtora* suppressors of RNA silencing 1 (PSR1) effector protein targets plant miRNAs and the PSR2 prevents the biogenesis of siRNAs (Qiao et al., 2013, Xiong et al., 2014).

A high abundance of pathogen siRNAs is not required to produce a significant effect on the host plant (Baulcombe, 2015). In studies where viruses induced gene silencing, a cascade is observed where viral siRNAs that have sequence homology to host RNA, can trigger secondary siRNA formation from the cleaved transcript. These secondary siRNAs can act as templates for RDR6-mediated dsRNA synthesis, causing amplification of the signal that leads to enhanced gene silencing of the target gene. Furthermore, the secondary siRNAs can experience diversification of the target mRNA, depending on the cleaved transcript upon which the RDR6 polymerase acts (Calo et al., 2012, McHale et al., 2013).

Plants use extracellular vesicles to send their assault sRNAs to the interacting pathogen. This was demonstrated in the *Arabidopsis-Botrytis* pathosystem, where sRNA profiling from *B. cinerea* protoplasts identified over 40 *A. thaliana* sRNAs. *Arabidopsis* vesicle sRNAs were isolated from the apoplastic fluid and profiled. These sRNA profiles coincided with the *Arabidopsis* sRNAs found in the *B. cinerea* cells. The study further demonstrated that these transferred sRNAs were protected by their extracellular vesicle packaging from nuclease digestion (Cai et al., 2018b). Observations in the *Arabidopsis-Pseudomonas syringae* pathosystem are congruent with this finding that sRNAs are transported in extracellular vesicles (Rutter and Innes, 2017). A recent study in *Arabidopsis* indicated that plant sRNAs are loaded into extracellular vesicles by RNA-binding proteins with differing efficiencies, suggesting a selectivity of sRNA translocation (He et al., 2021).

The cross-kingdom RNA interference phenomenon has been disputed by a recent study in the tomato-*B. cinerea* pathosystem (Qin et al., 2022). High-throughput sRNA sequencing of the interaction between tomato and *B. cinerea* in the early stage of infection (12 – 24 hours post inoculation) were compared to the transcription levels of the predicted target mRNAs. Indeed, a fraction of the target mRNAs was silenced. However, reducing the production of sRNAs in *B. cinerea* did not significantly reduce the pathogen's virulence. The production of sRNAs was obtained by generating *B. cinerea* mutants which either had a deletion in a sRNA-rich transposon region (responsible for the production of 10% of total sRNAs) or by deletion of two *dicer-like* genes, which reduced over 99% of transposon-derived sRNA that are 20–24 nt in length (Qin et al., 2022). The study claims that sRNAs produced by *B. cinerea* does not contribute much towards the virulence of the pathogen in tomato and that there is no functional evidence for cross-kingdom RNAi in this pathosystem. This is a surprising report

considering the many examples of cross-kingdom RNAi supported in previous literature. Moreover, previous studies have used the external application of dsRNA to target *B. cinerea* pathogenicity genes, including the genes for the Dicer-like proteins (DCL1 and DCL2), and demonstrated significant disease suppression in tomato and a wide range of fruits, vegetables, and flowers (Wang et al., 2016, Qiao et al., 2021) (see Section 2.2.6 for similar studies). A previous study which demonstrated naturally occurring cross-kingdom RNAi in tomato-*B. cinerea* provided evidence that some of the *B. cinerea* sRNAs that are transported to the host associate with plant AGO1 and that the plant target gene was downregulated (Weiberg et al., 2013).

In response to the findings of the tomato-*B. cinerea* sRNA analysis study, the researchers who performed some of the fundamental cross-kingdom RNAi studies in tomato-*B. cinerea* published their criticisms of the findings and the methodology (He et al., 2023). Among the criticisms published, He *et al.* highlight that the conidia concentration that Qin *et al.* used in their detached tomato leaf assay (1000 conidia/µL suspension in potato dextrose broth) was far more concentrated than a natural infection pressure and that the high concentration of inoculum masks the compromised virulence effects that could be observed by the deletion of *B. cinerea DCL1* and *DCL2* genes. When He *et al.* performed a repeat of this experiment they used lower conidia concentration (10 conidia/µL suspension in 1% malt extract) of the *dcl1/2* deletion mutants to provide a more accurate natural infection pressure and they observed significant reduction in lesion formation in the deletion mutants (He et al., 2023). In addition, He *et al.* disputed the claim made by Qin *et al.* that their deletion mutants eliminated all transposon-derived sRNAs. The sRNA sequence data which Qin *et al.* used to base their claims is not available in their NCBI repository, however when He *et al.* reproduced the study, they found that many retrotransposon-derived sRNAs were still present in the *B. cinerea dcl1/2* deletion mutants. Thus, in a detailed report provided by He *et al.*, the authors argued that the results obtained by Qin *et al.* cannot be relied upon based on the provided experimental evidence and interpretation of sequencing data (He et al., 2023).

2.2 Applications of RNAi

The many advances in RNAi research not only allowed for a better understanding of the mechanisms behind RNAi pathways, but also uncovered exciting findings indicating that sRNAs can be transferred between interacting organisms and can be taken up from the environment. These studies allowed for advancements in many fields of genetics, biotechnology, host-pathogen interactions, and crop protection strategies. In this section, various applications of RNAi will be discussed, with a specific focus on crop protection, starting with the early pioneering studies in *C. elegans*.

2.2.1 Early RNAi application studies

The roundworm *C. elegans* has frequently been used as a model organism in biological research and was also the organism where RNAi was first discovered (Guo and Kemphues, 1995). The first report of miRNA was produced from a study in *C. elegans* investigating *LIN* transcription factor genes, whereby they suggested that transcription of the *LIN-4* gene regulates the translation of *LIN-14* via an antisense RNA-RNA mechanism (Lee et al., 1993). Some of the earliest RNAi application studies were conducted on *C. elegans* (Fire et al., 1991). A study conducted on RNAi in *C. elegans* investigated the structures of RNA populations which are responsible for interference and found that some molecules were of double-stranded character and that dsRNA was significantly more effective (with at least two orders of magnitude) in interference than ssRNA, of either sense or antisense strands (Fire et al., 1998). Early studies which introduced exogenous RNA in the protozoan parasite, *Trypanosoma brucei*, were consistent with the dsRNA finding that both sense and antisense strands are required for efficient post-translational gene silencing (Ngo et al., 1998). During this time, early RNAi application studies applied exogenous dsRNA through microinjections in *C. elegans* to achieve target gene silencing (Fire et al., 1998, Montgomery et al., 1998). Applications of RNAi-mediated gene silencing in biotechnology have continued expanding since and have provided researchers with novel and exciting uses of RNAi.

2.2.2 Functional studies

Double-stranded RNA-mediated interference has been used to study gene function of coding regions in genomes. The use of sequence-specific sRNA constructs allows for targeted gene knockdown in functional genomics studies. This provides a simpler and faster reverse genetics approach compared to conventional methods such as random mutagenesis, which utilises chemical agents to generate mutants, or targeted mutagenesis approaches, such as homologous recombination (Jain et al., 2020). RNAi-mediated reverse genetics approaches are an elegant solution in organisms that are compatible with RNAi technologies. However, there are aspects of dsRNA-mediated interference which need to be considered in order to perform accurate functional gene studies. From their pioneering work in *C. elegans*, Fire *et al.* highlighted three such considerations: i) the possibility for interference in related genes with similar gene sequences exists, ii) genes with low levels of expression may escape this interference, and iii) there may be a small number of cells that will not be reached by the RNAi treatment (Fire et al., 1998).

2.2.3 Virus-induced gene silencing (VIGS) in plants

Viruses that infect plant hosts generate dsRNA intermediates during their replication and transcription of viral genes. These dsRNA products are cleaved into siRNAs by the host DCL proteins. The resulting siRNAs silence viral genes with sequence complementarity, reducing viral replication and virulence. This results in the inhibition of viral RNA translation and replication of RNA viruses (Baulcombe, 2015). This silencing mechanism has been adopted in a plant protection method known as virus-induced gene silencing (VIGS).

Early studies in RNAi observed co-suppression in transformed tobacco plants containing the transgene for the tobacco etch virus (TEV) coat protein. The transgenic plants were able to express the viral protein sequence and subsequently recovered 3-5 weeks after TEV inoculation. These experiments confirmed that the plants provided resistance specifically to repeat TEV infections and not to an unrelated control virus (Lindbo et al., 1993). Their molecular assays indicated that while TEV was actively replicating, the virus did not accrue mRNA for corresponding coat proteins, and they hypothesised that post-translational gene silencing was involved. Following this study, a strategy was developed which utilized vectors that express cDNA from replicating potato virus X (PVX) to deliver a reporter gene*, β-glucuronidase* (*GUS*), both in addition to and in place of the coat protein gene. However, instead of observing accumulated levels of the *GUS* transgene, high levels of post-transcriptional gene silencing was observed, as well as reduced levels of *GUS* expression and PVX infection in inoculated plants (Angell and Baulcombe, 1997). They also observed suppressed transiently expressed RNA, which shared sequence homology with the transgene, and concluded that this new strategy could provide a method for gene silencing in transgenic plants.

Waterhouse *et al.* demonstrated the use of dsRNA as the initiator of gene silencing in what can be regarded as one of the first VIGS studies. By using transgene expression of sense and antisense potato virus Y (PVY) gene sequences and combining the expression of these sequences through crossing transgenic plants, resistance to PVY infections was observed (Waterhouse et al., 1998). The parental lines which contained only sense or antisense PVY gene sequence did not provide protection against PVY infections. The expression of a hairpin RNA from an inverted repeat construct was also very effective at gene silencing, compared to the use of the sense or antisense transgenes individually (Waterhouse et al., 1998, Wang and Waterhouse, 2000).

VIGS studies can be used in functional genetics to elucidate the function of genes targeted by the sequences inserted into the viral expression vectors (Rössner et al., 2022). For example, a study which utilised VIGS to deliver miRNA sequences elucidated the role of the plant miRNAs, SlymiR157 and SlymiR156, and the key ripening gene, *LeSPL-CNR,* in the fruit ripening process in tomato (Chen et al., 2015a). In addition, VIGS has been used to deliver small guide RNA (sgRNA) sequences of the clustered regularly interspaced palindromic repeat (CRISPR)-Cas system in *Arabidopsis* and

Nicotiana tabacum to edit the plant genome (Baltes et al., 2014). This virus-induced gene editing system has been used to genetically edit a wide range of monocots and dicots (Ellison et al., 2020, Rössner et al., 2022).

VIGS utilises a viral expression vector to express transgenes in transgenic plants and therefore VIGS applications in plant protection could fall into the next category that will be discussed (Section 2.2.4), where gene silencing is induced by the plant host. Examples of studies that utilised VIGS to control plant pathogens are; wheat leaves infected with Barley Stripe Mosaic Virus (BSMV) strains that contain antisense sequences which target genes in *Fusarium culmorum* (Chen et al., 2016), and the use Tobacco Rattle Virus (TRV) constructs in transient expression in tomato plants to target *V. dahliae* transcripts (Song and Thomma, 2018).

2.2.4 Host-induced gene silencing (HIGS)

The discovery that sRNAs can transit between plant host and associated organisms allows for the potential development of novel crop protection strategies (Baum et al., 2007, Nowara et al., 2010, Baulcombe, 2015). One of these strategies is termed host-induced gene silencing (HIGS) and relies on dsRNA produced by the host plant that targets and silences specific virulence genes of the invading pathogen. These host plants are transgenic and early studies have been able to produce dsRNA against invading fungi to knockdown target virulence genes in cereal pathogens such as the maize fungal pathogen *F. verticillioides* (Tinoco et al., 2010), the cereal powdery mildew pathogen *Blumeria graminis* (Nowara et al., 2010), and the wheat leaf rust pathogen *P. triticina* (Panwar et al., 2013) (Table 1.2).

The exact mechanism of RNAi in HIGS is not known; whether the long dsRNA generated by the HIGS host plant is processed into siRNA in the host through plant RNA processing machinery or whether this process takes place in the associated fungus with fungal RNA processing machinery is unclear (Baulcombe, 2015, Hua et al., 2018). It is known, however, that plants package sRNAs in extracellular vesicles which are taken up by associated fungi, and these plant sRNAs are then processed by the fungal RNAi machinery (Wang et al., 2016, Cai et al., 2018b). It has also been shown that plant hosts send endogenous miRNA that enter fungal pathogens to silence fungal genes in the cotton – *V. dahliae* pathosystem (Zhao et al., 2016b). Research has also shown that sRNAs of fungal pathogens are sent to the host plant and processed by the plant's RNAi machinery where these fungal siRNAs act similarly to effectors. The sRNAs mediate the virulence of the fungal pathogen by silencing target genes in the host (Weiberg et al., 2013). Follow-up studies showed that fungal siRNAs are processed by fungal DCL proteins before being transported to their plant hosts (Wang et al., 2016). Yet, the possibility remains that fungal dsRNAs are transported to the plant hosts, where the plant DCL proteins process and produce the fungal siRNAs (Zhao et al., 2016a, Hua et al., 2018). Future studies could investigate whether it is possible for HIGS to send pre-processed siRNAs or long dsRNAs to fungal pathogens to mediate target gene silencing.

The fungal ergosterol biosynthetic pathway is targeted and disrupted with the use of commercial systemic fungicides, such as sterol demethylation inhibitors (DMI). DMI fungicides such as the triazole, propiconazole, can be found commercially in South Africa (Table 1.1); for example, as part of the active ingredients in Artea® (Syngenta, 2022). DMI fungicides bind to and inhibit the enzyme Cytochrome P450 lanosterol C-14α-demethylase (CYP51), causing disrupted fungal membrane integrity (Yoshida, 1993). Therefore, the ergosterol biosynthesis related *CYP51* gene has also been identified as an essential gene for the survival of the fungus. HIGS has been reported to successfully reduce *F. graminearum* disease symptoms in transgenic *Arabidopsis* and barley by targeting the ergosterol biosynthetic pathway (Koch et al., 2013). In this HIGS study, three paralogous fungal sterol 14α-demethylase (*CYP51*) genes were targeted by a single dsRNA construct in transgenic plants and the results showed almost complete resistance to *F. graminearum* infection. Table 1.3 provides a summary of some successful HIGS studies that demonstrated disease control of fungal plant pathogens.

A notable limit of HIGS strategies is that not all crop species can be efficiently transformed. Maize is an example of a crop that is not easily genetically engineered, with the highest transformation efficiency

of 18% reported for the Hi-II hybrid line (Vega et al., 2008, Yadava et al., 2017). In addition, there is still consumer resistance towards the use of genetically modified (GM) crops and commercialisation of GM crops is highly regulated in some countries, such as those within the European Union (Wunderlich and Gatto, 2015, Briefs, 2017, Kleter et al., 2018).

2.2.5 RNAi Insecticides

The mechanism of action of RNAi in animals differs from that of plants in a few respects, but successful applications of RNAi in pest control have also been studied for crop protection purposes (Millar and Waterhouse, 2005). The mode of action of the miRNA pathway in animals consists of translational repression, whereby the miRNA binds to the target mRNA of complementary sequence and inhibits translation and expression of the target gene (Mallory and Vaucheret, 2006, Eamens et al., 2008). Early studies in *C. elegans* demonstrated that ingested dsRNA efficiently silences target genes (Timmons and Fire, 1998), which provided a novel research perspective for pest management strategies to target plant-damaging insects.

Studies have reported on HIGS plants, which were transformed with a transgene for a specific dsRNA, to confer resistance to specific pests. Conventional genetically modified crops are transformed with the *Bacillus thuringiensis* insecticidal proteins (Bt toxins) to provide protection against Lepidopteran and Coleopteran insect pests (Romeis et al., 2006). HIGS strategies were utilised to express target gene-specific dsRNA and provided efficient protection against pests such as the western corn rootworm (Baum et al., 2007). This provided the first commercial HIGS product to target an insect pest, the maize hybrid MON 87411 which was developed by Monsanto Company (Bolognesi et al., 2012, Head et al., 2017). Other examples of successful dsRNA-mediated gene silencing in crop pests have been demonstrated in insects of the orders Coleoptera (beetles), Lepidoptera (moths and butterflies), and Hemiptera (aphids, psyllids, hoppers, cicadas, and stinkbugs) (Cooper et al., 2019, Christiaens et al., 2020).

2.2.6 Spray-induced gene silencing (SIGS)

Additional studies which have been conducted on the development of crop protection strategies using RNAi have made use of externally applied RNA to mediate gene silencing in what is referred to as spray-induced gene silencing (SIGS). These studies were preceded by findings that fungal pathogens, such as *B. cinerea, V. dahliae, S. sclerotiorum* and *Fusarium* spp., can take up external dsRNA which are then processed into sRNAs by fungal RNA silencing machinery. These sRNAs can silence targeted fungal pathogenicity genes of interest and cause disease resistance in the host plant (Koch et al., 2016, Wang et al., 2016, McLoughlin et al., 2018, Song and Thomma, 2018). Two pathways are suggested for fungal uptake of external dsRNA: either there is direct uptake of dsRNA by the fungi or dsRNA taken up by plant cells are transferred to fungal cells (Wang and Jin, 2017). SIGS studies in barley-*Fusarium* interactions demonstrated that the fungal DCL1 protein was required to process the dsRNA and for efficient gene silencing to occur (Koch et al., 2016).

SIGS strategies have been successfully applied in plants to protect against insects. The first report of external dsRNA foliar application was in citrus and grapevine trees to protect against two psyllids (*Diaphorina citri* and *Bactericera cockerelli*) and the sharpshooter (*Homalodisca vitripennis*) (Andrade and Hunter, 2016). There are many successful SIGS pest disease management reports. Examples are from studies in tomato against *Diabrotica virgifera*, in maize against the corn borer *Ostrinia furnacalis*, and in potato against the Colorado potato beetle *Leptinosa decemlineata* (Dalakouras et al., 2020). SIGS applications have also been studied in the protection against viruses; for example, in protection of maize plants against Sugarcane Mosaic Virus (SCMV). External dsRNA, which targets two fragments of a SCMV *CP* (coat protein) gene, effectively inhibited SCMV infection in maize (Gan et al., 2010).

2.3 SIGS as a crop protection strategy against fungi

2.3.1 SIGS fungal phytopathogens targets

Previous successful studies in the application of SIGS against fungal plant pathogens have targeted fungal genes which are essential to the virulence or pathogenicity of the pathogen (Table 1.3). One of the first reports of an RNAi-based plant protection strategy using external dsRNA application was on barley leaves to protect against *F. graminearum* infection (Koch et al., 2016). This study followed on their findings of HIGS experiments which targeted the fungal Cytochrome P450 lanosterol C-14α-demethylase gene (*CYP51*), involved in ergosterol biosynthesis. They combined three *CYP51* gene targets into a single 791 nt long dsRNA product which was then applied as a spray onto barley leaves. Fungal growth inhibition was observed in addition to target gene silencing and this study provided promising results for applications of SIGS as a means of crop protection.

A University of California research group has conducted extensive studies on external dsRNA application in the *Arabidopsis-Botrytis* pathosystem, investigating *Botrytis* pathogenicity genes such as *SAC1, DCL-1, DCL-2, VPS51,* and *DCTN1* (Wang et al., 2016, Cai et al., 2018b, Qiao et al., 2021) (Table 1.3). The *suppressor of actin-like phosphoinositide phosphatase* (*SAC1*) gene is involved in the regulation of vesicular trafficking, specifically secretory membrane trafficking (Liu and Bankaitis, 2010). The research group showed that a *B. cinerea sac1* deletion mutant strain had reduced virulence on the *A. thaliana* host, but not reduced growth in culture media. This suggests that *SAC1* has a direct role in fungal virulence and not simply the fungal growth (Cai et al., 2018b). In addition, this study created single deletion mutants for two other vesicle-trafficking pathway genes, *vacuolar protein sorting 51* (*VPS51*) and *dynactin* (*DCTN1*). These mutants had significantly reduced virulence on *Arabidopsis* leaves. The research group also used SIGS to target key components of the RNAi machinery in the fungus, such as genes responsible for DCL enzymes, *DCL1* and *DCL2*. SIGS-mediated gene silencing of fungal *DCL* genes has shown reduced disease severity in *B. cinerea* on various vegetables and plant structures (Wang et al., 2016, Qiao et al., 2021).

SIGS studies in the rapeseed (*Brassica napus*) – *S. sclerotiorum* pathosystem targeted essential fungal genes such as those involved in reactive oxygen species responses, transcription, and host colonization (McLoughlin et al., 2018). Gene silencing was obtained *in vitro* and reduced *S. sclerotiorum* lesions were observed *in planta* on two different host species, *B. napus* and *Arabidopsis*. A new dsRNA product was created, using *S. sclerotiorum* sequences that are homologous to the closely related *B. cinerea*, and SIGS reduced *B. cinerea* lesions by 66% in *B. napus* detached leaf assays (Table 1.3). Interestingly, some of the targeted homologues provided different levels of protection against the two pathogens. These findings provide exciting possibilities for the development of a single dsRNA construct which can target two different pathogen species infecting a single plant.

Table 1.3: Summary of previous studies that applied HIGS and SIGS against fungal pathogens and the genes which were targeted.

Method	Pathogen	Host	Gene target(s)	Results	Reference
HIGS	*F. graminearum*	Barley	Three *CYP51* genes	Transgenic lines showed almost complete resistance to infection	(Koch et al., 2013)
HIGS	*Blumeria graminis*	Barley and wheat	An effector gene (*Avra10*)	Reduced fungal development	(Nowara et al., 2010)
HIGS	*P. triticina*	Wheat	Genes for a MAP kinase (*PtMAPK1*), a cyclophilin (*PtCYC1*), and calcineurin B (*PtCNB*)	Up to 70% reduction in transcription of the endogenous target genes in infected wheat and significant disease suppression	(Panwar et al., 2013)
HIGS	*Aspergillus flavus*	Maize	*alpha-amylase* (*AMY1*)	Reduced *AMY1* expression, fungal colonization, and aflatoxin accumulation	(Gilbert et al., 2018)
HIGS	*F. graminearum*	Wheat	*chitin synthase* (*CHS*) *3b*	Significant reduction of lesion lengths and efficient down-regulation of target genes.	(Cheng et al., 2015)
SIGS	*F. graminearum*	Barley	*CYP51* genes involved in ergosterol biosynthesis	Inhibited fungal growth on treated and distal plant tissue	(Koch et al., 2016)
SIGS	*B. cinerea*	Fruits, vegetables, flowers, and *Arabidopsis*	*DCL1* and *DCL2*	Reduced disease by attenuating fungal pathogenicity and growth	(Wang et al., 2016)
SIGS	*B. cinerea* and *S. sclerotiorum*	*Brassica napus*	Three genes associated with reactive oxygen species	Reduced disease lesions	(McLoughlin et al., 2018)
SIGS	*B. cinerea, S. sclerotiorum. Aspergillus niger, R. solani, V. dahliae,* and *P. infestans*	Fruits, vegetables, and rose petals	*SAC1, VPS51,* and *DCTN1* targeted by a single dsRNA construct and another construct targeting *DCL1* and *DCL2*	RNA uptake efficiency varies across species and SIGS efficiency is dependent on the pathogen's dsRNA uptake ability. SAC1/VPS51/DCTN1 dsRNA treatment reduced disease symptoms, pathogen virulence, target gene mRNA expression levels, and fungal *in vitro* growth. DCL1/DCL2 dsRNA treatment reduced *B. cinerea* virulence and disease symptoms.	(Qiao et al., 2021)

2.3.2 Advantages of SIGS

An advantage of SIGS is the reduced risk of pathogen resistance development towards SIGS treatment, as opposed to resistance which is often reported to popular and conventional fungicides for the agricultural industry (Fisher et al., 2018). This is due to the nucleotide-specificity of this method and the evidence that gene silencing operates throughout the entire sequence length of the dsRNA construct. In addition, future SIGS applications could target multiple genes and pathogens through a single construct (Rosa et al., 2018). The use of biomolecules as a crop protection strategy has the added benefit of being biodegradable and eco-friendly. The SIGS strategies that combat crop pathogens target species-specific gene sequences and therefore it is safer to consume by animals and humans compared to currently available chemical fungicides.

It was observed in the dsRNA SIGS application in barley leaves that unsprayed distal leaf parts were able to confer resistance against *F. graminearum* infection, suggesting that dsRNA which was taken up by the plant had translocated to distal cells and tissues (Koch et al., 2016). This study also provided evidence that labelled RNAs were detected in xylem, phloem parenchyma cells, mesophyll cells, trichomes, and stomata. This provides another promising advantage of SIGS; that plant uptake of dsRNA can provide ample protection against invading pathogens, even if the entire plant was not treated with the dsRNA spray.

2.4 Challenges associated with the use of SIGS to control fungal pathogens

There are some challenges that need to be addressed when SIGS is to be considered as an efficient crop protection strategy. The longevity of the naked dsRNA on leaf surfaces is restricted and hence, plant protection will be time-limited. Northern blot assays in SIGS-treated barley leaves indicated the presence of efficient dsRNA 7 days post spray application, either through stable duration on the leaf surfaces or within the plant cells (Koch et al., 2016). Similar findings in *Arabidopsis* were reported where dsRNA, targeting *B. cinerea* genes, significantly decreased fungal growth for up to 7 days post application (Qiao et al., 2021). To navigate these limits, innovative studies have suggested the use of nanoparticles such as clay nanosheets (Mitter et al., 2017), carbon quantum dots (Taning et al., 2020), and lipid vesicles (Bochicchio et al., 2014) to protect and extend the longevity of RNA. High costs associated with dsRNA creation is another challenge for SIGS-based products which would be augmented by these innovative solutions to create a stable and viable product.

In SIGS strategies it is important that the targeted pathogen can successfully take up externally applied dsRNA efficiently, as not all plant pathogens have shown this ability or equal efficiency of dsRNA uptake. Examples of fungal phytopathogens with low or absence of external dsRNA uptake are: the wheat pathogen *Zymoseptoria tritici* (Kettles et al., 2019), the anthracnose causal agent *Colletotrichum gloeosporioides*, and the oomycete *P. infestans* (Qiao et al., 2021).

RNA interference off-target effects are possible because siRNAs are highly sequence-specific and not target gene-specific. Therefore, gene silencing effects can be observed in non-target genes that contain a degree of sequence complementarity to the siRNAs. A transcript profiling analysis in mammalian cell cultures has indicated that as few as 11 – 15 contiguous nucleotides in the transcript need to be identical to the siRNA molecule in order to obtain direct gene silencing. This study also found that there is a gene silencing bias of the 3'-end of the siRNA sense strand (Jackson et al., 2003). The lower limit of siRNA efficiency has not been determined in plant SIGS applications yet. However, the mammalian cell study informs that when designing SIGS constructs, the selected sequences for target gene silencing need to consider the genomes of the host plant and interacting organisms. The 3'-end of the construct's sense strand needs to be carefully evaluated for any sequences that could cause off-target gene silencing effects. Moreover, the sequences of both sense and antisense strands need to be evaluated since both could act as guide RNAs to perform gene silencing (Niu et al., 2021). These considerations have been applied successfully in various honeybee biosafety studies to ensure that these pollinators are not negatively affected by dsRNAs that target pathogens and parasites (Arpaia et al., 2021).

Off-target gene silencing could work in favour of certain RNAi-mediated crop protection applications. In cases where siRNAs can target multiple transcripts within the same gene family of the pathogen, increased gene silencing can lead to stronger disease control. This has been observed in *F. graminearum* where the dsRNA construct, targeting three ergosterol biosynthesis-related *CYP51* genes, exhibited the ability to silence other non-target fungal *CYP51* genes. The result of this off-target effect was that more efficient *Fusarium* resistance was obtained in barley, compared to the *CYP51* single-gene dsRNA constructs (Koch et al., 2019).

It is possible that there can be dsRNA exposure to humans and animals through direct contact or indirect exposure though the food chain. However, there are many factors involved in the physiological uptake of these dsRNAs. In order for dsRNA molecules to survive after ingestion, they would need to overcome various barriers such as RNA degradation prior to ingestion, breakdown of RNA by saliva and gastric enzymes, RNA degradation in the haemolymph, cellular uptake mechanisms, and the mode of RNA transport within the organism (Christiaens et al., 2018). In the case of human consumption of SIGS-treated plant material, it is unlikely that the dsRNA will be intact after encountering the digestive system. The dsRNA sequence specificity to genes of a chosen fungal crop pathogen also means that off-target effects in animals and their gut microbiota is highly unlikely.

The public perception of RNAi could provide difficulty to the acceptance of SIGS as a commercial crop protection product. Although SIGS application would not cause treated crops to fall into the genetically modified organisms (GMO) category, the public might become sceptical of the nature of SIGS when they are informed that specific genes are targeted by a complex genetic regulatory approach. However, SIGS has an advantage over HIGS strategies because SIGS does not involve host gene editing or gene modifications. sRNA-mediated gene silencing at post-transcriptional gene level is also a natural phenomenon in eukaryotes. Moreover, the effects of SIGS are reversible and not inherited by progeny. For these reasons, the SIGS technology is not expected to be subjected to GMO biosafety regulations; however, clear regulatory frameworks and risk assessment guidelines for SIGS products are not available yet (De Schutter et al., 2022). Externally applied dsRNA SIGS-based products are expected to be regulated as a biochemical pesticide (Dietz-Pfeilstetter et al., 2021).

3. Conclusion

There is an increasing need for global food supply and improved crop yields. The major world food, health, wildlife, and humanitarian organisations have collaborated to provide compelling reports on the importance of agriculture as a major driver in addressing future global food security issues, sustainability, and economic factors (FAO, 2022). Maize is a major crop and staple food globally, and the future production of maize crops is threatened by climate change and the effects of crop pathogens (Juroszek and Tiedemann, 2013). There are many advantages of using external RNAi instead of conventional chemical fungicides as a means for crop protection; i) dsRNA is present in all organisms and is biodegradable, ii) controlling crop diseases with external dsRNA application is safer to consume and is more environmentally friendly compared to use of chemical fungicides, iii) there is a lower risk of targeted pathogens to develop resistances towards a dsRNA strategy, and iv) innovations in SIGS can provide novel dsRNA constructs that can target multiple genes and pathogen species. The research and innovations in sustainable solutions are contributing to the development of resilient agricultural practices that can increase crop yields.

This Master's study will contribute towards research that develops crop protection technologies that improve food security. A previous study in our research group has indicated that *C. zeina* has the ability to successfully take up external dsRNA (Marais et al., 2019). A dsRNA construct was designed to target three pathogenicity genes in *C. zeina* (*CHSD/ECP2/PSD3*) and the *in vitro* studies indicated that this dsRNA construct successfully reduced the transcription of the target genes and reduced the fungal cell

viability by 34% (Marais, 2020). Thus, the research question for the current study is: can externally applied dsRNA technology be applied as a GLS disease control method in maize? It is hypothesised that the successful SIGS strategies reported against other fungal foliar pathogens can be used to reduce GLS disease symptoms in infected maize. The aim of the study is to reduce GLS disease symptoms in inoculated maize by applying the dsRNA construct which targets three *C. zeina* pathogenicity genes (*CHSD/ECP2/PSD3*). The following objectives were set for the study: i) to develop an external dsRNA application method in *C. zeina*-inoculated maize leaves, ii) to investigate if the timing of external dsRNA application affects the efficacy of dsRNA treatment in *C. zeina*-inoculated maize leaves, iii) to investigate the germination time of *C. zeina* conidia *in vitro* and *in planta* by using light- and scanning electron microscopy in order to consider the uptake of dsRNA in fungal structures, and iv) to perform pathogenicity trials in order to evaluate the ability of the applied dsRNA to suppress disease.

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Chapter 2

Gene silencing mediated by dsRNA reduces grey leaf spot disease in maize

Abstract

Grey leaf spot (GLS) is a significant foliar disease of maize and the causal agent for GLS in southern Africa is the fungal foliar pathogen *Cercospora zeina*. GLS is one of the most destructive maize diseases that causes devastating crop yield losses for commercial and small-holder farmers in sub-Saharan Africa and the USA. Advances in plant biotechnology have utilised natural gene silencing pathways to provide alternative crop protection products to harmful chemical fungicides. RNA interference (RNAi) is a phenomenon where small interfering regulatory RNAs (siRNAs) bind to and silence sequence-specific target gene transcripts. Next generation RNAi fungicides make use of external application of the precursors of siRNAs, long double-stranded RNAs (dsRNAs), in what is known as spray-induced gene silencing (SIGS). dsRNA uptake has been demonstrated by some plant pathogenic fungi, leading to successful SIGS studies conducted for crop disease control. Previous studies report that SIGS successfully mediated gene silencing, disease suppression, reduced lesion size, and fungal biomass in various fungal pathogens such as *Botrytis cinerea, Fusarium graminearum,* and *Sclerotinia sclerotiorum*. This study investigated whether dsRNA-mediated gene silencing could reduce GLS disease symptoms in maize leaves infected with *C. zeina.* The rationale for this study was based on previous findings that *C. zeina* has shown the ability to take up external dsRNA and that a dsRNA construct, 3Cz dsRNA, which targets three *C. zeina* pathogenicity genes, significantly reduced target gene expression *in vitro.* The results of the present study indicated that up to 39 – 56% of GLS disease symptoms were reduced by the application of 3Cz dsRNA in a growth chamber infection trial. The controls included inoculation with *C. Zeina* with and without a GFP-targeting dsRNA treatment, and these controls showed similar levels of GLS disease. Interestingly, when 3Cz dsRNA was applied at the same time as fungal infection, no significant GLS disease suppression was observed, compared to significant disease suppression when 3Cz dsRNA was applied 24 h after inoculation. Light microscopy and scanning electron microscopy (SEM) were used to investigate the initial infection stages of *C. zeina* and indicated that up to 77% of conidia were germinated within 24 h both *in vitro* and *in planta*. It is hypothesised that germ tubes of germinated conidia allowed for better uptake of the applied dsRNA and thus allowed for a more efficient RNAi-mediated gene silencing in the fungus, which successfully reduced GLS disease development. This study provides promising evidence that RNAi-based biotechnology methods can be used in future research to develop a safe, effective, and environmentally friendly product that protects maize crops from GLS disease.

Keywords: *Cercospora zeina*; GLS; maize; RNA interference; SIGS

1. Introduction

Crop pests and pathogens pose a major threat to global food security and agricultural production. Fungal pathogens are one of the most challenging obstacles in crop health management, leading to crop yield losses and reduced quality (Savary et al., 2019, Bebber and Gurr, 2015). Crop protection strategies typically include the use of commercially available fungicides. While practices such as crop rotation and plant breeding are often combined with fungicides, these methods are not equally effective in disease management or possible for all crops. In addition, reliance on chemical fungicides poses health risks and contributes to the ongoing emergence of fungicidal resistance in fungal pathogens (Fisher et al., 2018). There is a persistent need for a crop protection strategy which is environmentally friendly, effective, and safe for humans and animals.

Novel strategies in biotechnology are making advances in studies of plant pathology and have made use of natural endogenous genetic processing pathways to provide protection to valuable crops. RNA interference (RNAi) is the phenomenon where small RNA molecules, of typically 21-24 nucleotides (nt) in length, bind to mRNA transcripts through complimentary base pairing and mediate gene silencing by modifying the expression of target gene transcripts (Ghildiyal and Zamore, 2009). These

small interfering RNAs (siRNAs) are produced by the action of Dicer, an enzyme with RNA helicase and endonuclease activity, acting on long double-stranded RNA (dsRNA) (Bernstein et al., 2001). The siRNAs are bound by the Argonaute enzyme, which is contained within an RNA-induced silencing complex (RISC). siRNAs typically regulate target genes through transcript degradation or the inhibition of translation (Torres-Martínez and Ruiz-Vázquez, 2017).

Advances in crop protection strategies use accurately designed dsRNAs that target specific genes in the pathogen to reduce its virulence and pathogenicity (Salame et al., 2011, Taning et al., 2020). RNAi can be effected by these dsRNAs through the expression of a transgene in transformed plants in what is termed host-induced gene silencing (HIGS) (Baulcombe, 2015). HIGS provides constant generation of designed dsRNAs to provide consistent protection; however, HIGS is limited by the transformation ability of the crop species, the genetic stability of the transgene, and the restrictions on the use of genetically modified (GM) crop species in some regions (Koch and Kogel, 2014). Spray-induced gene silencing (SIGS) is a novel approach in crop protection strategies that can overcome these limitations. SIGS relies on the external application of dsRNA to plants, which can be taken up by the plant and the pathogen and processed by endogenous RNAi machinery, to silence selected pathogen genes and reduce disease. SIGS has been successfully applied in the protection of cereal crops such as barley against *Fusarium graminearum*, rice against *Rhizoctonia solani*, and the protection of fruits, vegetables and flowers against various fungal pathogens such as *Botrytis cinerea*, *Aspergillus niger*, and *Verticillium dahliae* (Koch et al., 2016, Wang et al., 2016, Koch et al., 2018, Qiao et al., 2021). External dsRNA treatment was also shown to reduce *Sclerotinia sclerotiorum* lesion size and disease severity in canola where up to 59% of target gene silencing was obtained (McLoughlin et al., 2018).

Cercospora zeina Crous & Braun is a fungal foliar pathogen which causes grey leaf spot (GLS) disease in maize in southern Africa (Crous et al., 2006, Meisel et al., 2009). GLS is a major destructive disease of maize leaves, leading to poor crop health and reported yield losses of up to 60% (Ward et al., 1999). *C. zeina* is a hemibiotrophic facultative pathogen with a long latent period (Ward et al., 1999, Crous et al., 2006). Warm (> 20 °C) and humid (> 90% relative humidity) environmental conditions enhances GLS pathogenicity (Beckman and Payne, 1982, Nowell, 1997). During winter, the fungal conidia stay present in the soil which enables the pathogen to infect the following season's crops (Ward, 1996, Ward et al., 1999, Nsibo et al., 2019). There is limited knowledge on the infection strategy of *C. zeina*. Instead, inferences are made from infection and disease development studies conducted on its close relative, *C. zeae-maydis*. *C. zeae-maydis* conidia germinate 24 h after inoculation (Beckman and Payne, 1982). Germ tubes grow towards the stomata and form an appressorium four to five days post inoculation (dpi). The appressoria form penetration pegs, and penetration commences six to seven dpi through the leaf stomata and subsequently leads to the establishment and colonisation of fungal hyphae in the intercellular spaces of the leaves. Fungal colonisation is limited by sclerenchyma tissue surrounding the major veins, which causes the characteristic long, rectangular lesions of GLS (Beckman and Payne, 1982). These lesions cause loss of the plant's photosynthetic capabilities, leading to reduced crop yields (Ward et al., 1999). The necrotrophic phase leads to host cell death, increased fungal biomass, and conidiogenesis (Caldwell and Laing, 2005).

Previous work has shown the successful uptake of exogenously applied dsRNA by the *C. zeina* fungus *in vitro*. Fluorescently-labelled dsRNA was used to show dsRNA uptake by the conidia, mycelia, and protoplasts of *C. zeina* (Marais et al., 2019, Marais, 2020). This RNAi research in *C. zeina* confirmed genes can be silenced by externally applied dsRNA. A dsRNA construct, 3Cz dsRNA, which targets three *C. zeina* genes was developed and reduced the *in vitro* fungal viability by 34% compared to the negative control treatments (Marais, 2020). The three genes targeted by this single construct are*: extracellular protein 2 effector* (*ECP2*), *chitin synthase D* (*CHSD*), and *phophatidylserine decarboxylase proenzyme 3* (*PSD3*).

The aim of this study was to show that externally applied dsRNA can reduce GLS disease symptoms in maize leaves which have been inoculated with *C. zeina*. Here, the effect of dsRNA applied to maize leaves that were inoculated with *C. zeina* was investigated. It was shown that dsRNA which targets three pathogenicity genes in *C. zeina* can successfully reduce GLS disease symptoms. This study reports that efficient GLS disease suppression was observed when dsRNA was applied one day after *C. zeina* infection. How and when *C. zeina* conidia germinate *in planta* was assessed using histological methods. In summary, it was found that *C. zeina* conidia germinate within 24 h of infection on the maize leaves, potentially enabling greater uptake and thus efficacy of externally applied dsRNA.

2. Materials and Methods

2.1 Fungal cultures

Cercospora zeina isolate CMW 25467 (Meisel et al., 2009) was used as wild-type strain in this study. *C. zeina* isolates were routinely maintained as 15% (v/v) glycerol stocks and kept at -80 °C. To obtain conidia, *C. zeina* was cultured on V8 agar medium supplemented with 50 µg/mL Cefotaxime (Beckman and Payne, 1982) in ø 60 mm x 15 mm plates for seven days before the conidia were isolated. Conidia were isolated by washing the culture with 1.5 mL double-distilled water (ddH_2O) and scraping the culture with a stainless-steel spatula.

2.2 Maize plants

Maize (*Zea mays*) seeds from inbred line B73 were planted in a mixture of seedling mix potting soil (Culterra, Johannesburg, South Africa) and river sand in a ratio of 2:1 (w/w). The plants were grown at 22 °C ± 6 °C in a Conviron PGC Flex 1 Tier plant growth chamber (Conviron, Winnipeg, Canada) with a day/night cycle of 13/11 h and 60% relative humidity (RH). The growth chambers were programmed to emit different light intensities throughout each 24-hour cycle. The average maximum midday readings were 148 µmoles/m^2/s. These conditions were programmed based on weather data collected (AccuWeather, 2022) for the Cedara region (KwaZulu-Natal, South Africa) where maize fields are severely affected by GLS (Ward et al., 1994). The plants were irrigated daily with 600 mL water and fertilised every second day with 600 mL 2 g/L of Solu-Cal Plus (Solu-Cal, USA) and Hygroponic (Hygrotech, Pretoria, South Africa) fertilisers in a 1:1 (w/w) ratio.

2.3 Synthesis of dsRNA

A recombinant pJet1.2/blunt plasmid, named pJet1.2_3Cz dsRNA, containing a 1.403 kb sequence that targets regions in three pathogenicity genes in *C. zeina* namely, *CHSD* (798 bp), *PSD3* (404 bp), and *ECP2* (201 bp) was used as a template to produce 3Cz dsRNA (Fig. S1). This 3Cz dsRNA construct was designed and tested *in vitro* in a previous study by our research group (Marais, 2020). The 3Cz dsRNA was used as the test treatment in the current study. A recombinant pJET plasmid, pJet1.2_gpdAsGFP, containing a 325 bp sequence which targets a region in the *Aequorea victoria* green fluorescence protein (*GFP*) gene was used as a template to produce GFP dsRNA (Fig. S2). The GFP dsRNA served as a negative dsRNA treatment control in this study to show that the dsRNA effect on GLS disease was not a non-specific effect of dsRNA. The previous MSc study (Marais, 2020) demonstrated that GFP dsRNA does not silence *C. zeina* genes and does not reduce fungal cell viability *in vitro*. Thus, the GFP construct was chosen as a dsRNA specificity negative control in this study.

Gene-specific primer pairs (Table 2.1) were designed (Marais, 2020) to PCR amplify the construct sequences from the plasmids and to add T7 RNA polymerase promoter sequence overhangs to the amplified PCR products for use in *in vitro* transcription reactions. The primers contain T7 promoter sequences at each 5'-end. Primers used in this study were synthesised by Inqaba Biotech, Pretoria, South Africa. The PCR reactions contained 10mM of the forward (F) and reverse I primers, 10 ng plasmid, 3 µl of 1X Ampliqon DNA Polymerase Master Mix RED (Odense, Denmark) and ddH_2O to a final volume of 10 µl. The PCR reaction conditions included an initial denaturation step at 95 °C for 2

min, 30 cycles of 95 °C, primer annealing temperature, and 72 °C for 30 seconds each, and final extension step at 75 °C for 2 min. The primer annealing temperature is 59 °C to produce GFP PCR products and 72 °C to produce 3Cz PCR products (Table 2.1). The PCR reactions were performed with a GeneAmp® PCR 2700 System thermocycler (Applied Biosystems, Foster City, California, USA. The PCR products were subjected to electrophoresis in a 1.2% agarose gel (w/v), prepared with SeaKem agarose (Lonza, Basel, Switzerland) and 50 mL 1X TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, and 2 mM Na_2.EDTA.2H_2O) and stained with ethidium bromide (EtBr) [0.1 µg/mL final concentration]. The PCR products were subsequently purified from the agarose gel using a Zymoclean™ Gel DNA recovery kit (Zymo Research, Irvine, USA) and then used as templates to synthesize GFP and 3Cz dsRNAs using the HiScribe® T7 high yield RNA synthesis kit (New England Biolabs, Ipswich, Massachusetts, United States). The transcription reaction contained 1 µg of template DNA, 10 µL nucleoside triphosphate buffer mix (final concentration of 10 mM for each nucleoside triphosphate), 2 µL of T7 RNA polymerase mix, and nuclease-free water to a total reaction volume of 20 µL. The reaction was incubated at 37 °C for 2 h in a GeneAmp® PCR 2700 System thermocycler. Template DNA was removed by the addition of 30 µL of nuclease-free water and 2 µL of Dnase I to the reaction mixture, followed by incubation for 15 min at 37 °C. The dsRNA products were purified using the NucleoSpin™ RNA kit (Macherey-Nagel, Fisher Scientific, Düren, Germany). All of the above procedures were performed in accordance with the manufacturers' instructions. Prior to agarose gel electrophoresis, the dsRNA was heated at 94 °C for 5 min and then allowed to reanneal at room temperature to remove any potential secondary RNA structures. Aliquots of the dsRNA products were then subjected to agarose gel electrophoresis (performed as described earlier) and the bands were visualised using a ChemiDoc (Bio-Rad Laboratories, Hercules, California, United States) gel imaging system (Fig. S3). The molecular weight markers used were 100 bp and 1 kb Fast DNA ladders (New England Biolabs). DNA concentrations were determined with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, United States).

Table 2.1: Primers used in this study.

Target and function	Primer name and sequence	Tm (°C)	Expected size of product	Reference
Amplifies the *GFP* gene in pJet1.2_gpdAsGFP and adds T7 overhangs	T7-GFP forward (F): **TAATACGACTCACTATAGGG**CAAGGAGGACGGCAACATC T7-GFP reverse (R): **TAATACGACTCACTATAGGG**CTTGTACAGCTCGTCCATGC	59	365 bp (inclusive of 40 bp T7 overhangs)	(Marais, 2020)
Amplifies the 3Cz dsRNA sequence in pJET1.2_3Cz dsRNA and adds T7 overhangs	T7-CSD F: **TAATACGACTCACTATAGGG**CCATGACCGACTACCCAAATAG T7-PSD R: **TAATACGACTCACTATAGGG**GAAGCCCTCGACATTGACAT	72	1443 bp (inclusive of 40 bp T7 overhangs)	(Marais, 2020)

Primer sequences underlined and in bold indicate the T7 RNA polymerase sequence overhang. Tm indicates the primer melting temperature.

2.4 SIGS trials

To test whether the 3Cz dsRNA construct could reduce GLS disease *in planta*, *C. zeina* CMW 25467 conidia, 3Cz dsRNA, and GFP dsRNA were used in this study. A total of five different treatments were applied in this study: i) *C. zeina* conidia (a positive control for GLS disease), ii) *C. zeina* conidia mixed with 3Cz dsRNA (the test construct), iii) *C. zeina* conidia mixed with GFP dsRNA (the dsRNA specificity negative control), iv) 3Cz dsRNA applied 24 h after *C. zeina* inoculation, and v) GFP dsRNA applied 24 h after *C. zeina* inoculation.

The *C. zeina* conidia were collected in an ice-cold Falcon tube and suspended in a solution of 0.02% (v/v) Tween20 (Sigma-Aldrich/Merck, Darmstadt, Germany) in ddH_2O. The conidia were not filtered prior to inoculation on maize leaves. Fungal conidia were counted under an Olympus CX21 compound light microscope (Olympus Corporation, Shinjuku City, Tokyo, Japan) using a Neubauer-improved haemocytometer counting chamber (Marienfeld & Company, Munich, Germany). Conidia were mixed to a final concentration of 6×10^5 conidia/mL for the first trial and 7.95×10^5 conidia/mL in the second trial. The number of leaf collars were counted and used to determine the vegetative growth stages of maize (Ritchie et al., 1992). Seven-week-old maize plants with seven leaf collars (V7 leaf stage) were inoculated with *C. zeina* conidia mixed with 0.02% (v/v) Tween20. A single leaf was chosen at similar height from each maize plant. The inoculated leaves were positioned from the third to fifth leaf from the soil. A 10-cm section along the length of the leaf and starting 10 cm from the base of the leaf was marked with a marker pen. An artist's round paintbrush was used to inoculate 330 µL of the *C. zeina* conidia suspension to the top and bottom of the demarcated leaf area. The maize plants were left in darkness for 3 h prior to and after inoculation. The relative humidity of the chamber was between 80 – 85% for a week post inoculation to aid fungal infection.

The two dsRNA treatments consisted of GFP dsRNA (the dsRNA specificity negative control) and the 3Cz dsRNA (test construct). After synthesis, the dsRNA was mixed with RNase-free ddH_2O to a concentration of 33 ng/µL (66.38 µM) for the 3Cz dsRNA and 15 ng/µl (63.32 µM) for the GFP dsRNA. The dsRNA treatments were applied either at the same time as the *C. zeina* conidia inoculations or they were applied 24 h after inoculation. For immediate dsRNA application, the dsRNA products were mixed with the *C. zeina* conidia suspension just before inoculations took place. The second set of dsRNA treatments was performed by applying the dsRNA products to maize leaves which were inoculated with *C. zeina* the previous day. A total volume of 330 µL dsRNA was applied to the top and the bottom of the demarcated leaf surface areas with an artist's paintbrush. Four plants were used as biological replicates per dsRNA treatment and six plants served as positive controls that contained only *C. zeina* conidia inoculum.

2.5 GLS lesion evaluation

The GLS disease progression was evaluated at the start of lesion development and recorded every three days using the GLS disease score S1-S8. S1 denotes the absence of symptoms, and the score increases incrementally as foliar GLS symptoms develop to the most severe GLS symptoms of coalesced necrotic lesions (S8) (Table 2.2). Images of leaf samples were photographed with a Nikon D90 (Nikon, Minato City, Tokyo, Japan) digital single-lens reflex camera (DSLR) in natural light, without flash, on automatic camera settings, and at a 105 mm focal length.

Table 2.2: Grey leaf spot (GLS) disease scores. Disease scores were monitored and recorded for GLS disease symptom development on a scale ranging from absence of symptoms (S1) to the most severe symptoms of necrotic lesions (S8).

Score	GLS Symptom
S1	No symptoms
S2	Chlorotic spots (less than five spots)
S3	Initiation of a single lesion
S4	Initiation of two lesions
S5	Single tan lesion
S6	Two tan lesions
S7	More than two tan lesions
S8	Coalesced necrotic lesions

2.6 *In vitro* germination study

C. zeina was cultured on V8 agar culture media supplemented with 50 µg/mL Cefotaxime, as described previously (Section 2.1). After seven days, conidia were harvested with ddH_2O from plates. The conidia suspension was filtered through a double layer cheesecloth to remove fungal mycelia. The filtered suspension of 7.58 x 10^4 conidia/mL was added to three 1.5-mL Eppendorf® tubes (Eppendorf, Hamburg, Germany). The suspension was taken as the first time point (0 h) and samples from the three replicates were investigated under light microscopy (LM), and the number of germinated conidia and the total number of conidia was determined using a haemocytometer. Aliquots of the suspension were added to V8 agar culture media plates, creating at least three replicates for four time intervals: 3 h, 7 h, 16 h, and 24 h. Fungal conidia were harvested after the specified time intervals from the V8 agar plates using a sterilised stainless steel spatula and ddH_2O. The number of germinated and total conidia counts were recorded for the different time intervals and for each treatment (water and V8 agar media) using LM and a haemocytometer. Conidia collected from V8 agar media samples were investigated under an Olympus CX21 light microscope and images of conidia were captured and processed using Zeiss ZEN core® v.3.5 software (Carl Zeiss Microscopy, Jena, Germany).

2.7 *In planta* germination study

Maize leaves were inoculated with a suspension of *C. zeina* conidia in 0.02% (v/v) Tween20, as described above (Section 2.4), to a final concentration of 5.7 x 10^5 conidia/mL. At least three leaves were inoculated for three biological replicates of the following time intervals: 0 h, 16 h, 24 h, and 4 dpi. At each time interval, three leaves were collected, each from a different replicate, and eight 1-cm^2 sections were cut out with a sterilised scalpel blade from the inoculated areas. The eight square samples of each replicate were evenly distributed along the inoculated area (10 cm in length). The samples were immediately immersed in a fixative solution of 2.5% glutaraldehyde/formaldehyde (50% v/v) for at least 24 h. At least two samples were prepared for scanning electron microscopy (SEM) from three replicates of each time point to ensure that both adaxial and abaxial leaf surfaces are visualised. The samples were washed in a 0.75 M phosphate buffer three times for 15 min each and then subjected to an ethanol dehydration series consisting of 30%, 50%, 70%, and 90% (v/v) ethanol for 15 min each. The samples were subsequently dehydrated in 100% ethanol, twice for 15 min each, and finally for 30 min. The samples were then submerged in a mixture of hexamethyldisilazane (HMDS) and 100%

ethanol (1:1 v/v) for 1 h. Following incubation, the samples were submerged in 100% HMDS for 1 h, after which the samples were covered in fresh HMDS and left to dry overnight. Samples were mounted onto aluminium stubs, exposing an abaxial and adaxial leaf surface per replicate. Samples were coated in carbon using a Quorum Q150T Plus turbomolecular pumped coater (Quorum Technologies, Lewes, United Kingdom). The samples were visualised, and images were captured using a ZEISS 540 Gemini Ultra Plus FEG SEM (ZEISS) situated at the Laboratory for Microscopy and Microanalysis, University of Pretoria (Pretoria, South Africa). SEM images were captured of visible conidial structures on leaf samples. Two adaxial and two abaxial surfaces were inspected per biological replicate per time point. The germination percentage was calculated as the total number of germinated conidia divided by the total number of conidia observed within all of the SEM images of multiple replicates captured per time point multiplied by 100.

2.8 Statistical analysis

All of the experiments and data analysed for statistical significance were performed with at least three biological replicates. GraphPad Prism version 5.0 software was used to perform the statistical Mann-Whitney U test and to draw box plots. A two-tailed Mann-Whitney U test (Mann and Whitney, 1947) with Gaussian approximation was used to analyse the mean area under disease progress curve (AUDPC) scores (Van der Plank, 1963) and *in vitro* germination results in the SIGS experiments. Microsoft Excel (Microsoft Corporation, Albuquerque, New Mexico, United States) was used to draw the line graphs and charts.

3. Results

3.1 Synthesis of dsRNA products

The *in vitro*-synthesized GFP and 3Cz dsRNA products were viewed on a 1.2% agarose gel (Fig. S3). The GFP dsRNA band corresponded to the expected size of 365 bp (Table 2.2), whereas the 3Cz dsRNA band corresponded to a size of approximately 766 bp instead of 1443 bp (Table 2.2). Nevertheless, a 3Cz dsRNA product of approximately 766 bp was expected as this is in agreement with results reported previously (Marais, 2020). The smaller size of the 3Cz dsRNA product may be attributed to several reasons, such as secondary RNA structure formation that influences migration through the agarose gel or alternative primer binding sites within the 3Cz sequence yielding a shorter dsRNA product. In that study, the 3Cz dsRNA product was sequenced using gene-specific primers which are specific to the ends of the dsRNA sequence. The result showed that only 350 bp of the 798 bp of the first gene (*CHSD)* was synthesised, while the full length of *PSD3* (404 bp) and *ECP2* (201 bp) sequences were synthesised. The cause for the shorter dsRNA product is likely due to an unintended primer binding site within the sequence. Despite the shorter dsRNA product, the *in vitro* study showed that the 3Cz dsRNA product reduced mRNA transcripts of the target gene and significantly reduced the fungal cell viability by 34% (Marais, 2020).

3.2 The 3Cz dsRNA targeted to *C. zeina* pathogenicity genes reduces GLS disease *in planta*

The ability of dsRNA to reduce GLS disease was evaluated for the dsRNA test construct that targets three pathogenicity genes of *C. zeina* (3Cz dsRNA) in a SIGS pathogenicity trial. Maize leaves that were inoculated with *C. zeina* conidia served as a positive GLS disease symptom control. Leaves that were inoculated with *C. zeina* conidia and treated with GFP dsRNA served as a dsRNA specificity negative treatment control. The test treatment consisted of *C. zeina*-inoculated leaves that received 3Cz dsRNA treatment. Leaves that received dsRNA treatment had dsRNA applied either at the same time as the *C. zeina* inoculum or 24 h after inoculation (1 dpi). A GLS disease score (Table 2.2) was developed to record and compare the GLS disease progress observed in the positive disease control and the dsRNA treatments.

The first signs of GLS disease symptoms in the positive disease control maize leaves were visible as the development of small chlorotic spots (S2) on the inoculated areas (Fig. 2.1). The first signs of GLS lesions appeared as single (S3) or two (S4) small yellow rectangular shapes on the leaves, followed by single (S5) or two (S6) tan elongated rectangular lesions.

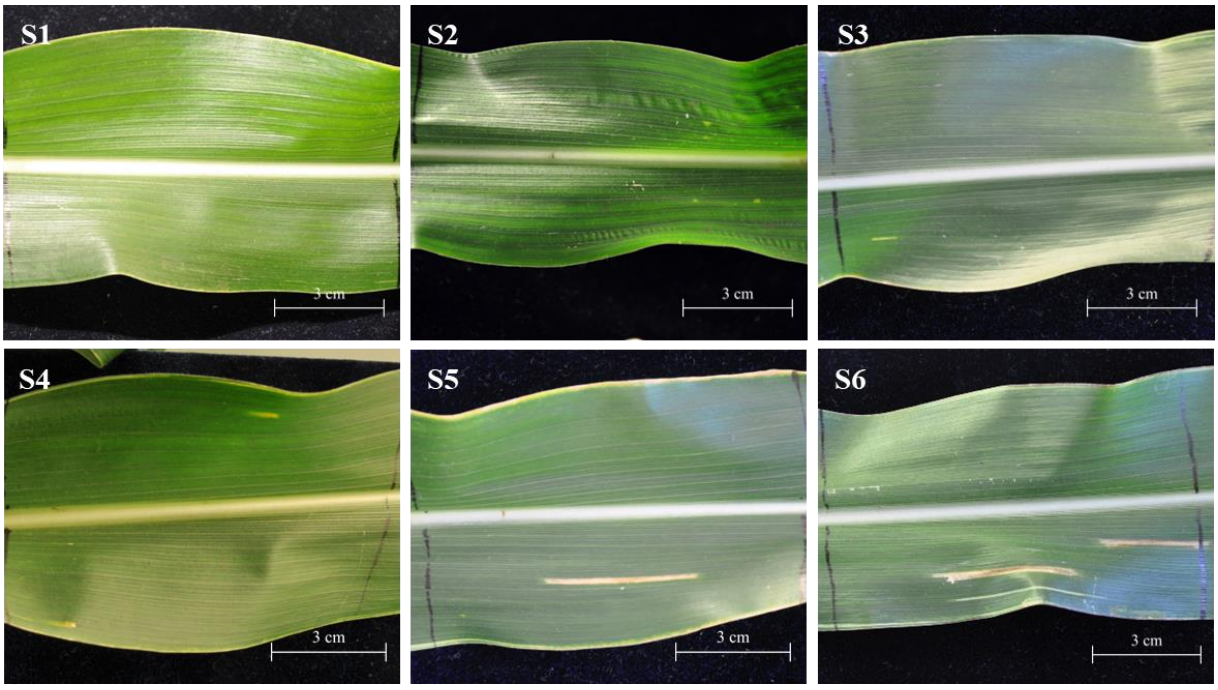

Figure 2.1: Representative images of the progression of GLS disease symptoms. Inoculated maize leaves were monitored, and GLS disease symptom development was recorded and scored according to the S1-S8 scale (Table 2.1). In the positive disease control leaves, GLS symptoms developed from absence of symptoms (S1) to two tan lesions (S6).

In the initial stages of GLS disease symptom development, the S2 – S3 symptoms developed sooner in the second trial compared to the first trial (Fig. 2.2). In the first trial, S2 symptoms developed 22 days post inoculation (dpi), S3 symptoms were visible by 28 dpi, and S4 symptoms were visible by 34 dpi. In contrast, a mixture of S2, S3, and S4 symptoms were observed by 13 dpi in the second trial. The S5 and S6 symptoms in the first trial developed at a similar time after inoculation compared to the symptom development in the second trial. S5 and S6 symptoms were frequently observed by 34 dpi (first trial) and 38 dpi (second trial).

The *C. zeina*-inoculated leaves which were treated with 3Cz dsRNA at 1 dpi showed a significant reduction in GLS disease development compared to the positive disease control of *C. zeina*, and the GFP dsRNA specificity negative control (Fig. 2.2). The GFP dsRNA-treated leaves showed a similar level of GLS disease symptoms compared to the *C. zeina* disease positive control (Fig. 2.2).

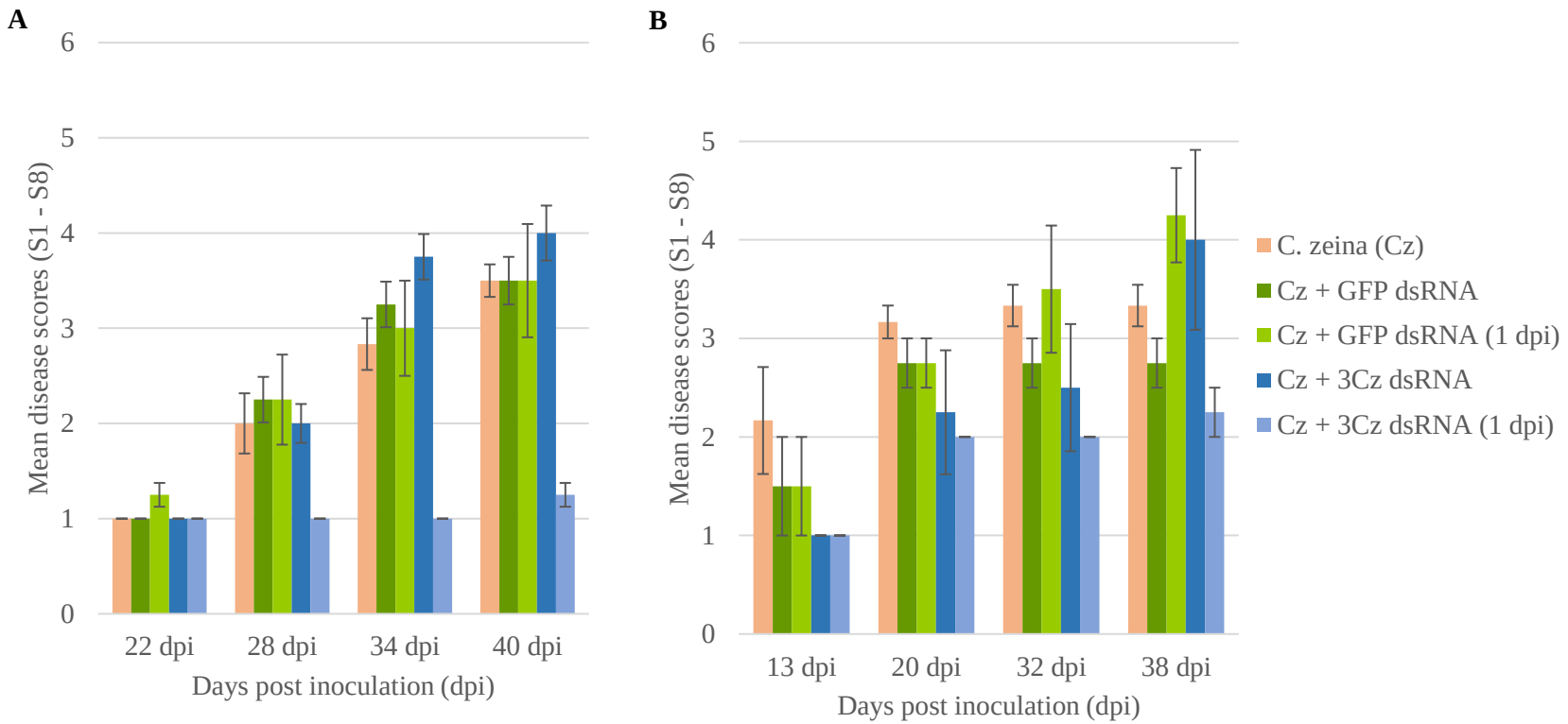

Figure 2.2: Mean GLS disease scores recorded in the first (A) and the second (B) SIGS trials. GLS disease symptoms were recorded weekly from the first signs of disease development at 22 (**A**) and 13 (**B**) days post inoculation (dpi), until 40 (**A**) and 38 (**B**) dpi when GLS lesions were mature. Treatments include *C. zeina* positive disease control; inoculated leaves treated with GFP dsRNA (dsRNA specificity negative control); inoculated leaves treated with GFP dsRNA at 1 dpi; inoculated leaves treated with 3Cz dsRNA (targeted towards three pathogenicity genes of *C. zeina*); and 3Cz dsRNA treatment applied at 1 dpi. Disease scores ranged from absence of symptoms (S1) to two tan lesions (S6). The 3Cz dsRNA treatment added at 1 dpi was the most effective treatment to reduce GLS symptoms in both trials. Standard error bars are indicated on the graph. Mann-Whitney U test ($\alpha = 0.05$) determined that no statistical significance was found between different data points.

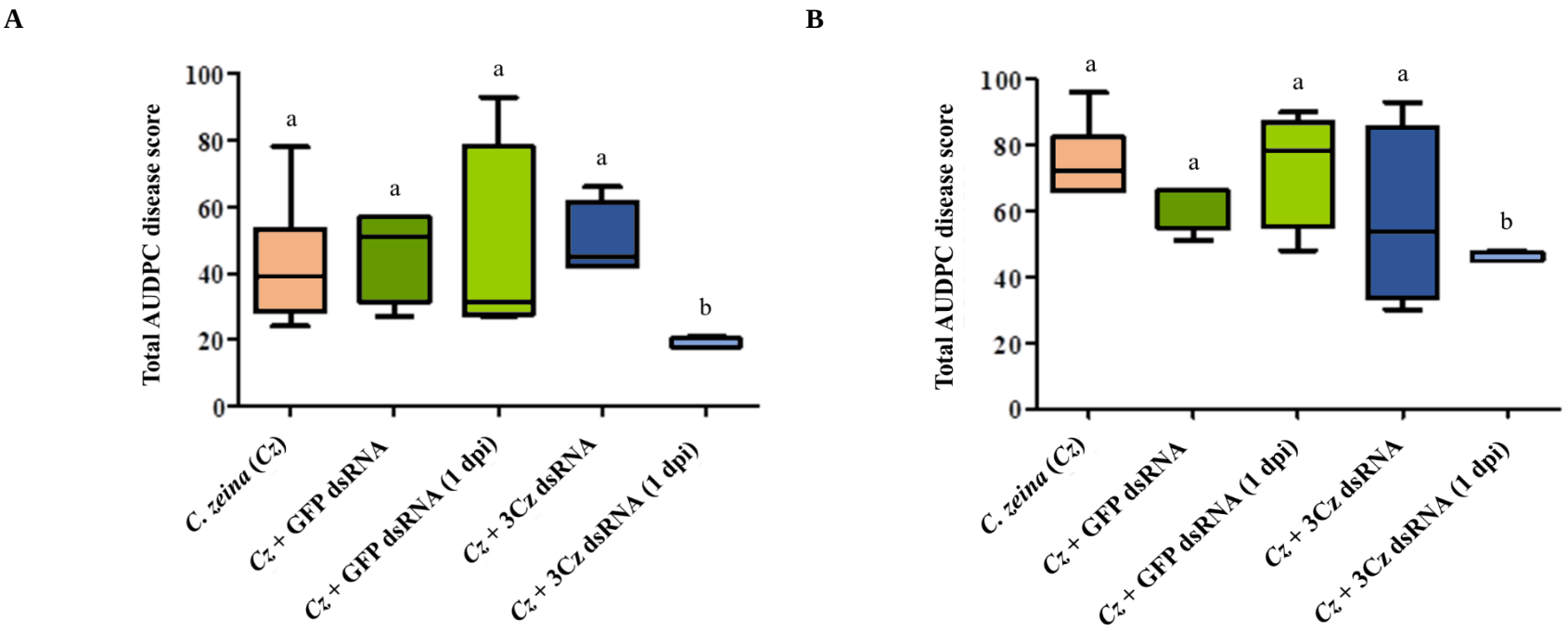

Figure 2.3: Box plots indicating the total area under disease progress curve (AUDPC) scores of the treatments used *in planta* on GLS-inoculated maize from the first (A) and second (B) trial. The treatments included *C. zeina* (*Cz*) control; *Cz* treated with GFP dsRNA (dsRNA specificity negative control); *Cz* treated with GFP dsRNA at 1 dpi; *Cz* treated with 3Cz dsRNA (the test construct targeted towards three pathogenicity genes of *C. zeina*); and *Cz* treated with 3Cz dsRNA at 1 dpi. In both trials, inoculated maize leaves treated with 3Cz dsRNA at 1 dpi showed significant GLS disease reduction compared to the positive disease control and the other treatments. Statistical significance between treatment means is indicated with letters (a, b) where the same letter indicates no statistical significance between two treatment groups, as determined by Mann-Whitney U test ($\alpha = 0.05$). Box plot whiskers indicate the minimum and maximum values.

The total area under disease progress curve (AUDPC) scores were calculated for each treatment in both SIGS trials and the AUDPC means were compared for statistical significance (Fig. 2.3). In both trials, the 3Cz dsRNA applied at 1 dpi was the only treatment that significantly reduced GLS disease symptom development compared to all other treatments, as determined by the Mann-Whitney U statistical significance test ($\alpha = 0.05$) (Fig. 2.3). The mean AUDPC scores obtained from the 3Cz dsRNA treatments applied at 1 dpi were 18.75 in the first trial and 45.75 in the second trial (Table S1). In contrast, the mean AUDPC scores of the *C. zeina* disease positive control were 42.5 and 75 in the first and second trial, respectively. The 3Cz dsRNA thus reduced GLS disease symptoms by 56% and 39% in the first and second trials, respectively. The mean AUDPC scores of treatments in which 3Cz dsRNA was mixed with *C. zeina* inoculum just prior to infection, was similar to the mean AUDPC scores of the *C. zeina* disease control and the two GFP dsRNA specificity negative control treatments (Fig. 2.3 and Table S1).

GLS disease symptoms increased in severity over time for all treatments, except for inoculated leaves which received 3Cz dsRNA treatment applied at 1 dpi (Fig. 2.2). The leaves which received the 3Cz dsRNA treatments applied at 1 dpi remained mostly symptomless, with some replicates showing small chlorotic spots. Some replicates of leaves that received the 3Cz dsRNA treatment which was applied at the same time as fungal inoculation, showed visible GLS disease suppression (Fig. S4). However, the total mean AUDPC disease scores of this treatment were not significantly different from the means of the treatments that received either GFP dsRNA or no dsRNA (Fig. 2.3).

3.3 *C. zeina* conidia germinated within 24 h *in vitro* and *in planta*

The percentage of *C. zeina* conidia that germinated was determined *in vitro* and *in planta* over various intervals. For *in vitro* studies, the fungal conidia were cultured in water as a control and on V8 agar culture media to provide nutrition. Conidia were harvested from V8 agar media plates containing mature *C. zeina* cultures and this first suspension (0 h) contained a low percentage of germinated conidia, with a mean germination percentage of 6.5% (Fig. 2.4). In four time intervals over a 24 h period, the number of germinated conidia observed increased in the V8 agar media treatments. In contrast, the mean percentage of germinated conidia grown in water was increased at the 3 h, followed by a smaller increase at 7 h, and subsequent measurements recorded similar germination percentages up until the last measurement. Conidia grown on V8 agar media showed a significantly higher percentage of germinated conidia at each time point compared to conidia grown in water ($\alpha = 0.05$). At 24 h of incubation, the mean germination percentage of the conidia grown in water was 33.6% and that of conidia grown on V8 agar culture media was 78.7% (Table S2).

Following the *in vitro* observations that 78.7% of *C. zeina* conidia germinate within 24 h of growth on V8 culture media, maize leaves were inoculated with a conidial suspension and samples were collected to determine if most conidia also germinate within 24 h *in planta*. Conidial germination was evaluated in inoculated maize leaf samples at different time intervals over 4 dpi (Fig. 2.5). Various SEM images of tissue samples were collected, and images were captured of visible *C. zeina* conidia. A total of 123 images across four time points were analysed to determine the percentage of germinated conidia observed at each time interval. For samples collected immediately after inoculation, 42.8% of conidia had germinated. This increased to 70.6% at 16 h after inoculation and by 24 h after inoculation, 77.2% of conidia on the maize leaves had germinated (Table S3). The percentage of germinated conidia remained approximately constant after 24 h, with 77.3% of conidia germinated at 4 dpi.

Light microscopy (LM) images were captured of conidia germinating *in vitro* isolated from V8 agar media and scanning electron microscopy (SEM) images were captured of inoculated maize leaf samples (Fig. 2.6). Less than half of the observed conidia were germinated at the first isolation (0 h) from V8 agar media plates (Fig. 2.6 i). The ungerminated conidia demonstrated the characteristic tapered shape with zero to four septa visible. SEM images of samples collected 0 dpi also showed an abundance of ungerminated conidia, mixed with conidia exhibiting short germ tubes (Fig. 2.6 ii). Germinated conidia

often exhibited more than one germ tube, extending either laterally or from the terminal end, both *in vitro* and *in planta* (Fig. 2.6 iii and 2.6 iv). Approximately 19 of the 149 (12.8%) observed germinated conidia from SEM samples showed germ tubes growing towards plant stomata (Fig. 2.6 iv).

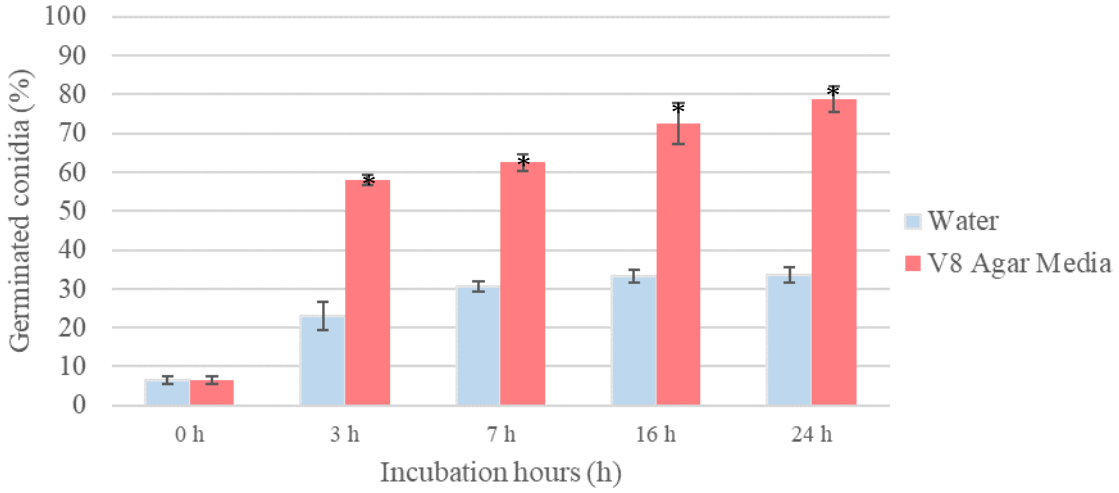

Figure 2.4: The percentage of germinated *C. zeina* conidia *in vitro* on V8 agar culture media and in water. The recorded mean percentage of germinated fungal conidia on V8 agar culture media increased over four intervals for 24 h. The percentage of germinated conidia in water increased until 7 h and then remained at a similar percentage until the last measurement at 24 h. Conidia grown on V8 agar showed a higher percentage of conidial germination compared to conidia grown in water. Statistical significance between water and V8 agar treatments at each individual time point was determined by the Mann-Whitney U test ($\alpha = 0.05$) and is indicated with an asterisk (*).

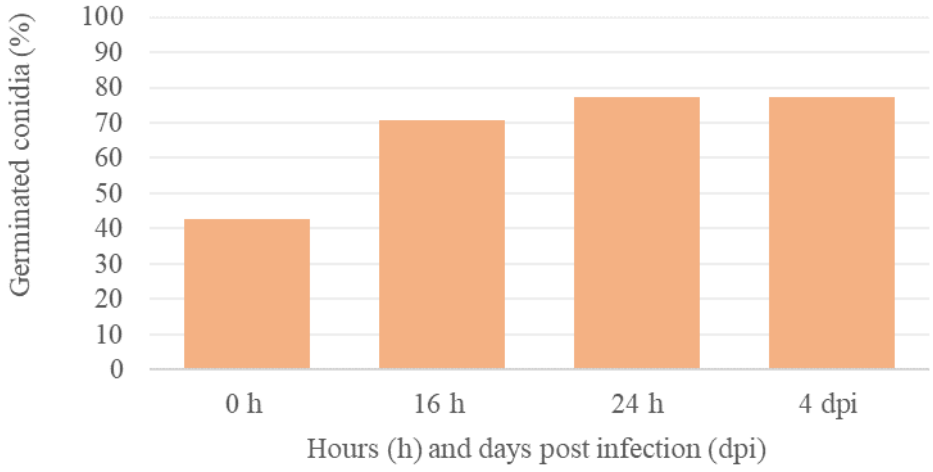

Figure 2.5: The percentage of germinated conidia observed on representative images of SEM samples. The percentage of germinated conidia observed on a total of 123 representative SEM images of inoculated maize leaf samples, which were collected at the time points of 0 h, 16 h, 24 h, and 4 days post inoculation (dpi), is shown.

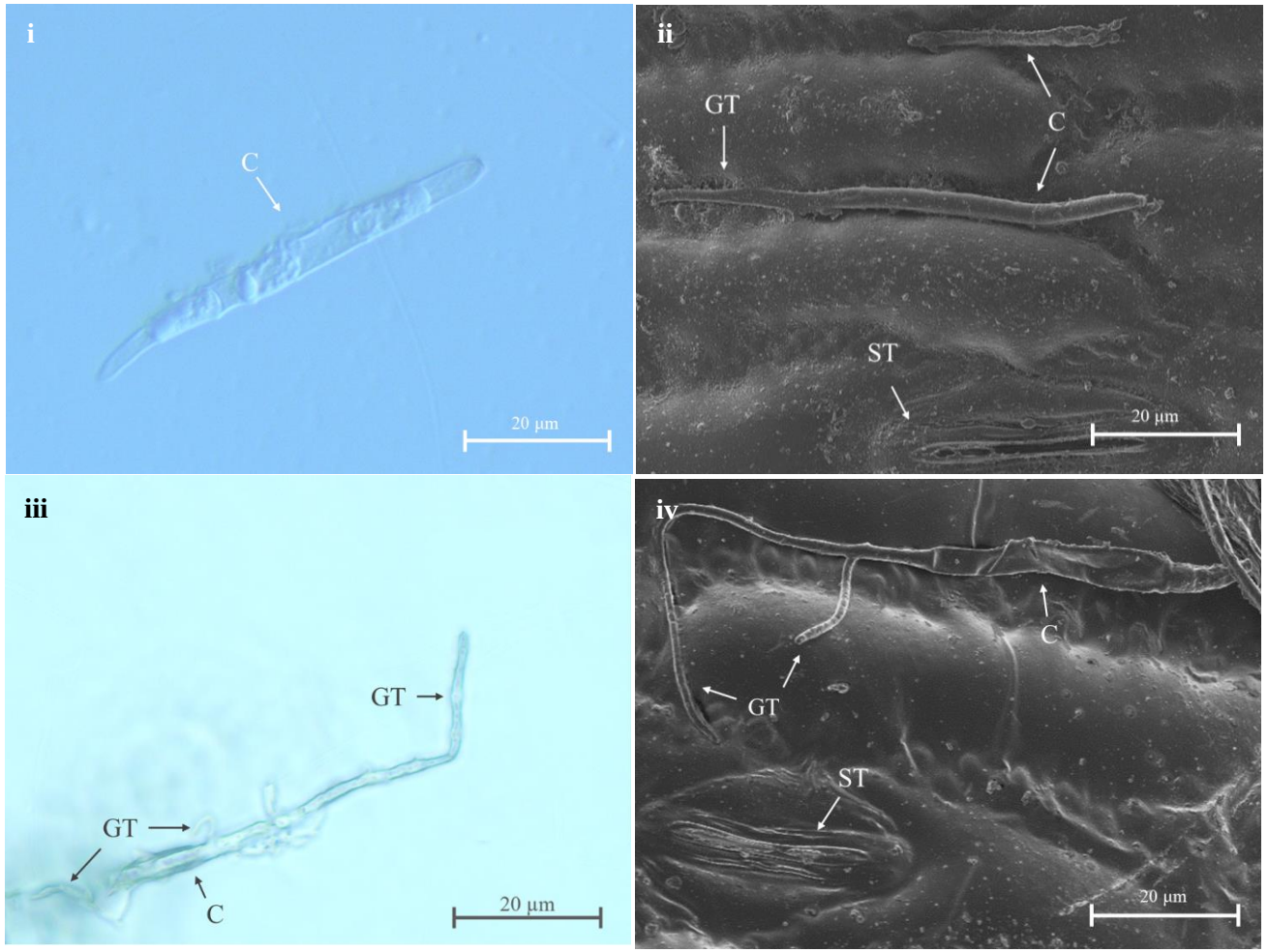

Figure 2.6: Light microscopy (LM) and scanning electron microscopy (SEM) images representing observations of germinating *C. zeina* conidia. LM images (**i, iii**) represent conidia grown on V8 agar culture media *in vitro*. SEM images (**ii, iv**) represent conidia grown on maize leaves *in planta*. Most of the conidia (C) were not germinated 0 h after isolation *in vitro* **(i)** and 0 h after infection on maize leaves **(ii)**. Germ tubes (GT) extended from germinated conidia. Up to 77% of conidia were germinated within 24 h *in vitro* **(iii)** and *in planta* (**iv**). SEM images indicated that growing germ tubes showed positive tropism towards the plant stomata (ST). **Abbreviations: conidium (C), germ tube (GT), and plant stoma (ST).**

4. Discussion

SIGS has been demonstrated as an effective disease management strategy in several fungal plant pathogens such as *B. cinerea*, *F. graminearum*, and *S. sclerotium* (Koch et al., 2016, McLoughlin et al., 2018). This study explored whether gene silencing mediated by dsRNA could be used to control GLS disease in maize. It was demonstrated that the application of external dsRNA targeting *C. zeina* pathogenicity genes can reduce GLS disease symptoms by as much as 39 – 56%, compared to the GLS infection control. Thus, this study supports the potential for RNAi to control fungal pathogens in crops and shows promising potential of SIGS as a GLS disease control strategy in maize.

The *C. zeina* GLS symptoms in the growth chamber studies took slightly longer to develop compared to previous glasshouse studies which reported latent periods in *C. zeina* (Meisel et al., 2009, Meyer et al., 2017) and in *C. zeae-maydis* (Beckman and Payne, 1982). Initial GLS symptoms started to develop on maize leaves as chlorotic spots between 13 – 22 dpi in this study, which is similar to previous reports of 14 dpi in *C. zeina* (Meyer et al., 2017), and 9 dpi in *C. zeae-maydis* (Beckman and Payne, 1982). GLS tan rectangular single lesions were first observed between 28 – 32 dpi in this study compared to previous reports of 19 dpi (Meisel et al., 2009) and 24 – 28 dpi (Meyer et al., 2017) in *C. zeina*, and 12 – 16 dpi in *C. zeae-maydis* (Beckman and Payne, 1982, Ringer and Grybauskas, 1995). The GLS lesions in this study reached their greatest surface area and started to mature and turn darker in colour around 34 – 40 dpi (Fig. 2.1 and Fig. 2.2). This contrasts with reports that necrotic coalesced GLS lesions in *C. zeae-maydis* developed around 16 – 21 dpi. The discrepancy in the time it takes for GLS disease symptoms to mature between our study and reports in *C. zeae-maydis* is possibly due to differences in latency periods between these species, as it has also been illustrated that *C. zeae-maydis* has a faster growth rate on artificial media (Crous et al., 2006). The discrepancy between the GLS latent periods in our study and previous studies in *C. zeina* could be due to differences between environmental conditions created in our growth chambers and in the other glasshouse studies. Our growth chamber trials simulated the environmental conditions of maize fields in Cedara, South Africa (AccuWeather, 2022), where maize fields are affected by devastating GLS disease infestations (Ward et al., 1994). Our growth chamber trials did not result in the severe coalesced necrotic GLS lesions that are observed in infected maize fields. *Cercospora* spp. are known to have long biotrophic phases before switching to the necrotrophic phase (Meredith, 1970, Beckman and Payne, 1982). The latent period observed before onset of symptoms is likely due to *Cercospora's* extended period of mycelial growth on the leaf surface before penetration occurs. *C. zeae-maydis* has been reported to sustain mycelial growth long after initial host penetration and lesion formation, initiating new penetrations and lesion formations (Rathaiah, 1977, Beckman and Payne, 1982).

In this study, the GLS disease progress was monitored in inoculated maize leaves that were treated with 3Cz dsRNA targeting the pathogen, leaves that were treated with GFP-specific dsRNA as a negative treatment control, and leaves that were not treated with external dsRNA. Our results indicate that inoculated maize leaves in the disease positive control developed similar GLS symptoms to the inoculated leaves which were treated with GFP-specific dsRNA as expected, with no significant total disease score differences observed between these treatments (Fig. 2.3). Interestingly, when dsRNA which specifically targets *C. zeina* pathogenicity genes (3Cz dsRNA) was applied, the timing of dsRNA application yielded different GLS disease results in inoculated leaves. When the 3Cz dsRNA was mixed with *C. zeina* inoculum immediately prior to maize leaf infection, the leaves developed GLS symptoms which were not significantly reduced compared to the other control treatments. However, when 3Cz dsRNA was applied a day after *C. zeina* infection, the maize leaves showed a significant reduction in GLS disease symptoms (Fig. 2.2 and Fig. 2.3).

Little is known about the infection biology of *C. zeina* and studies in *C. zeae-maydis* are used as reference for the infection strategy of *C. zeina*. Based on this study's results of dsRNA application *in planta*, we were motivated to explore the initial stages of *C. zeina* infection on maize leaves by using light- and scanning electron microscopy to observe conidial germination. The *C. zeina* conidia structure

development was investigated for the first 24 h and few days after inoculation. The microscopy results from this present study showed that *C. zeina* conidia germinate on maize leaf surfaces within 24 h and that some germ tubes show positive tropism towards the nearest stomatal openings (Fig. 2.6 iv). These results are consistent with previous reports that *Cercospora* germ tubes grow towards stomata to infect the host and that this tropism is largely dependent on the environmental conditions (Beckman and Payne, 1982).

Histological studies in *C. zeae-maydis* indicated that in the presence of free water, germ tubes readily germinated but showed limited attraction towards stomata, appressorium formation was rare, and no penetration was observed (Beckman and Payne, 1982). In contrast, positive germ tube tropism was mostly observed on the abaxial leaf surfaces and in the absence of free water (unmisted leaf areas) (Beckman and Payne, 1982). Studies conducted on *Cercospora beticola* on sugar beet and *Cercospora musae* on banana foliage suggested that germ tubes were likely attracted to stomatal openings due to hydrotropism (Meredith, 1970, Rathaiah, 1977). In addition, histological studies of *Cercospora* isolates from GLS-infected maize fields in the Cedara region indicated that the maximum amount of conidia released from the conidiophores occurred in between the hours of 12:00 – 14:00, during environmental conditions of high temperatures, increasing vapour deficits, and the lowest level of leaf wetness (Caldwell and Laing, 2005). However, observations of GLS disease in South African maize fields show that the presence of high humidity is also crucial for the virulence of the fungus when pertaining to; i) the development of conidia, which are preceded by incidences of high humidity, ii) germ tube elongation, which will not take place in conditions of relative humidities below 95%, and iii) the latency period, which can be decreased by high humidity levels in conjunction with high temperatures (Ward et al., 1999).

This study demonstrates that GLS symptom development was more effective when dsRNA was applied a day after *C. zeina* infection, compared to dsRNA application at the time of fungal infection. Our histological findings indicate that between 77 – 78% of *C. zeina* conidia germinated within 24 h *in vitro* (Fig. 2.4) and *in planta* (Fig. 2.5). It is hypothesised that increased germination counts could provide better uptake of applied dsRNA and explain the increased RNAi efficiency observed. Growing *C. zeina* germ tubes could consist of structural cell wall polymer composition which is different to that of fungal conidia and provide a more efficient route for dsRNA uptake. This hypothesis is congruent with qualitative microscopy observations from a previous study that *C. zeina* mycelia readily take up external fluorescently labelled dsRNA, compared to more selective dsRNA uptake by the conidia (Marais, 2020). Differential cell wall composition has been reported in the hyphae and infection structures of fungi such as the maize anthracnose pathogen *Colletotrichum graminicola*. It was proposed that the differential β-1,3-glucan and chitin composition in the cell walls is a mechanism that *C. graminicola* uses to evade plant defence responses to pathogen-associated molecular patterns, such as the oligomers found in degraded fungal cell walls (Oliveira-Garcia and Deising, 2013). This is consistent with the reports of surface modification of fungal cell walls in *C. graminicola*, and the rust fungi *Puccinia graminis* F. sp. *tritici* and *Uromyces fabae*, by converting exposed chitin to chitosan in order to avoid plant defence responses (El Gueddari et al., 2002). Thus, it is possible that dsRNA application to *C. zeina* germ tubes could enhance the RNAi-mediated gene silencing in the fungus and provide better protection against GLS. In contrast, application of dsRNA at the same time as infection with mostly ungerminated conidia, could lead to less uptake of dsRNA at the time of application, leaving dsRNA susceptible to degradation by RNases found in the environment.

RNAi-based strategies such as SIGS provide an efficient yet safe biocontrol alternative to harmful chemical fungicides which can be overcome by fungicidal resistance in pathogens (Fisher et al., 2018). The natural RNAi pathway has been used in the evolutionary arms race between host and pathogen in a phenomenon known as cross-kingdom RNAi, where the host sends small RNAs to silence target mRNAs in the pathogen to become resistant (Weiberg et al., 2013, Wang et al., 2016). In response, pathogens have evolved silencing suppressors to overcome host resistance conferred by natural RNAi

pathways (Qiao et al., 2013, Rosa et al., 2018). If suppressors of RNAi emerge within the targeted pathogen, the dsRNA construct could be adapted by including a sequence which would target the mRNAs of the silencing suppressor component, in addition to other targets of pathogenicity genes, to accomplish disease control. It is possible that pathogens could develop resistance to various degrees to treatments of different gene targets and in certain RNAi applications. For example, a fraction of resistance-breaking plant viruses has been observed in RNAi-based transgenic plant lines (Rosa et al., 2018). However, it is unlikely that pathogens could easily develop resistance to externally applied dsRNA, since the dsRNA sequences exhibit silencing effects throughout the entire target sequence and one or two mutations occurring within these sites would not greatly reduce the RNA silencing effect of the dsRNA construct (Niehl et al., 2018).

SIGS methods that utilise long dsRNAs provide additional benefits such as the ability to produce a great variety of siRNAs that enhance gene silencing effects, and a construct which can target multiple genes and even potentially target multiple pathogens. Due to the gene-specific nature of RNAi-based crop protection strategies, there is less risk of off-target effects, and the products are safe for human consumption and not harmful to non-target organisms (Arpaia et al., 2021). SIGS offers a favourable perspective on the use of natural genetic pathways to protect crops, without the need for genetic modification of the host and thus without additional GM crop regulations. Limitations of SIGS applications include the current costs associated with dsRNA production and the longevity of dsRNA in field conditions. SIGS-mediated crop protection strategies are not applicable to all types of crop pests and pathogens and with regards to fungal pathogens, it has been reported that the efficacy of SIGS mostly depends on the dsRNA uptake ability of the fungus (Qiao et al., 2021). This present *in planta* study, in combination with the previous *in vitro* study on RNAi-mediated gene silencing effects on *C. zeina* (Marais, 2020), provides proof-of-concept evidence for promising and novel solutions to GLS disease control in maize.

The results of this study suggest that a long dsRNA product could be designed to provide more efficient target gene silencing and thus provide even stronger GLS disease control. The previous findings of RNAi in *C. zeina* showed that the 3Cz dsRNA significantly reduced the relative gene expression levels of only one of the target genes, *chitin synthase D* (*CHSD*) (Marais, 2020). This *CHSD* gene is responsible for the synthesis of chitin, an essential fungal cell wall structural component, which makes it an attractive target for the development of fungicides (Latgé, 2007, Lenardon et al., 2010). The previous *in vitro* RNAi study in *C. zeina* showed a reduction of *CHSD* expression of up to 50% on mRNA level (Marais, 2020). Other pathogenicity gene targets could be considered for the development of future dsRNA constructs. For example, the fungal ergosterol biosynthetic pathway is targeted and disrupted with the use of commercial systemic fungicides, such as sterol demethylation inhibitors. These fungicides bind to and inhibit the Cytochrome P450 lanosterol C-14α-demethylase (CYP51), causing disrupted fungal membrane integrity (Yoshida, 1993). Therefore, the ergosterol biosynthesis-related *CYP51* gene has also been identified as an essential gene for the survival of the fungus, and a study in *Fusarium graminearum* successfully reduced disease symptoms through HIGS in transgenic *Arabidopsis* and barley (Koch et al., 2013, Koch et al., 2016).

Previous successful studies in SIGS application on fungal plant pathogens have targeted fungal genes which are essential to the virulence or pathogenicity of the pathogen. For example, studies on SIGS application in the *Botrytis – Arabidopsis* pathosystem were mediated by dsRNA and investigated pathogenicity genes such as *SAC1*, *DCL1*, *DCL2*, *VPS51*, and *DCTN1* (Wang et al., 2016, Cai et al., 2018, Qiao et al., 2021). The Suppressor of actin (SAC1)-like phosphoinositide phosphatase enzyme is involved in regulation of vesicular trafficking, specifically secretory membrane trafficking (Liu and Bankaitis, 2010). The research group demonstrated that a *B. cinerea* deletion mutant strain for a *SAC1* gene showed reduced virulence on the *A. thaliana* host, but not reduced growth in media. This suggests that SAC1 enzyme has a direct role in fungal virulence and not simply the fungal growth (Cai et al., 2018). In addition, this study created single deletion mutants for two other vesicle-trafficking pathway genes, *vacuolar protein sorting 51* (*VPS51*) and *dynactin* (*DCTN1*). The results indicated that these

mutants had significantly reduced virulence on the *Arabidopsis* leaves. The researchers also used SIGS to target key components of RNAi machinery in the fungus, such as genes responsible for Dicer-like (DCL) enzymes, DCL1 and DCL2. SIGS-mediated gene silencing of fungal *DCL* genes has shown reduced disease severity in *Botrytis cinerea* on various vegetables and plant structures (Wang et al., 2016, Qiao et al., 2021).

4.1 New dsRNA construct for future studies

Based on the above studies, a new dsRNA construct was designed in the present study to target some of these promising genes. An NCBI Basic Local Alignment Search Tool protein-protein (BLAST-p) search (NCBI, 2022) was performed on the amino acid sequences from the target proteins in *B. cinerea study* (Cai et al., 2018), and orthologous proteins were identified in *C. zeina* and other closely related *Cercospora* species. A new dsRNA construct was designed to target the *SAC1* (Fig. S5), *VPS51* (Fig. S6), and *DCTN1* (Fig. S7) gene sequences at areas which are highly conserved between *Cercospora* species. Highly conserved sections between orthologous amino acid sequences were selected as target sequences to ensure that a gene sequence is targeted which is less likely to undergo mutations and this will aid to the efficiency of the dsRNA construct's gene silencing effect. The sequences were selected towards the 3'-end of the gene sequence. This construct is provisionally named *SAC1/VPS51/DCTN1* and was synthesised by IDT (Integrated DNA Technologies, Coralville, Iowa, USA) and provided in a plasmid, pUCIDT_SVD (Fig. S8). The new dsRNA construct includes 175 bp of gene sequence per target gene, yielding a total size of 525 bp in length. Future experiments using RNAi to supress *C. zeina* disease in maize will target *CHSD* in addition to new genes.

The length of the gene fragments was decided based on previous studies which successfully achieved disease control with SIGS technology, such as the study in which the *CYP51* gene was targeted in *F. graminearum* and gene fragments of 200 – 300 bp were selected to create a single long dsRNA (791 bp) construct (Koch et al., 2018). A recent study has shown that increased sizes of long dsRNA (400 – 1500 nt) constructs can reduce the dsRNA uptake and thus efficacy in controlling the fungal pathogen *F. graminearum* in barley (Höfle et al., 2020). A study in western corn rootworm (*Diabrotica virgifera virgifera*) showed that dsRNA of at least 60 bp in size was required to reach biological activity. This study also highlighted that a 240 bp dsRNA containing a single 21 nt match was sufficient to knockdown target gene expression (Bolognesi et al., 2012). The literature available on how dsRNA size relates to its RNAi ability is largely conducted on insect and animal studies, with inconsistent conclusions with regards to how small dsRNA constructs can still be effective in RNAi. Thus, the length of the new dsRNA construct designed in this study was calculated based on the length of gene fragments which would generate many siRNAs and the ability of the fungus to take up large dsRNAs. It would be interesting to test this new dsRNA construct in future studies with the aim to obtain highly effective SIGS results in *C. zeina* which produce almost no GLS symptoms in maize.

To conclude, dsRNA-mediated gene silencing was more effective at GLS disease suppression when applied at 1 dpi compared to application at the time of fungal infection. Our histological studies showed that over 77% of *C. zeina* conidia germinate within 24 h. Germinated conidia and infection structures could provide a more effective method of dsRNA uptake by the fungus compared to ungerminated conidia. External application of dsRNA was able to significantly supress GLS disease. Continued studies in RNAi-based technologies can provide significant contributions to future studies in plant pathology and crop protection. RNAi-mediated strategies such as SIGS shows promising prospects in maize crop protection products against pests and pathogens. This study contributes to the foundational research to develop an efficient, environmentally safe, and non-transgenic RNAi-based fungicide to control crop pathogens such as GLS disease in maize.

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Appendix

Supplementary Figures

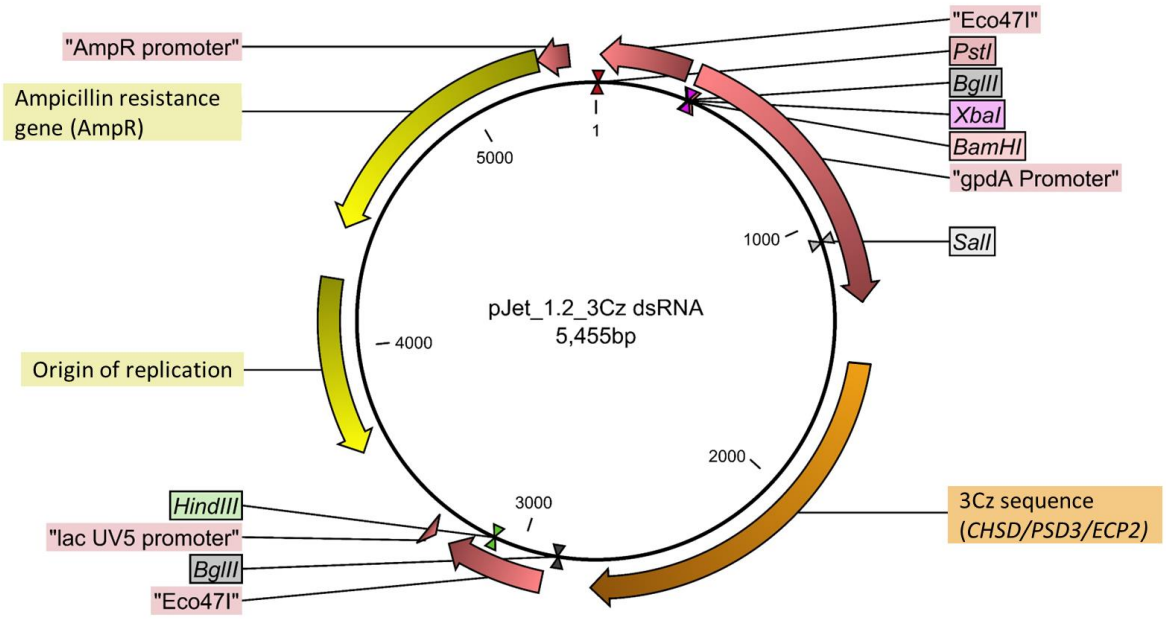

Figure S1: pJET_1.2_3Cz dsRNA plasmid map. The 3Cz (*CHSD/PSD3/ECP2)* sequence was cloned into the pJET1.2/blunt vector (Marais, 2020).

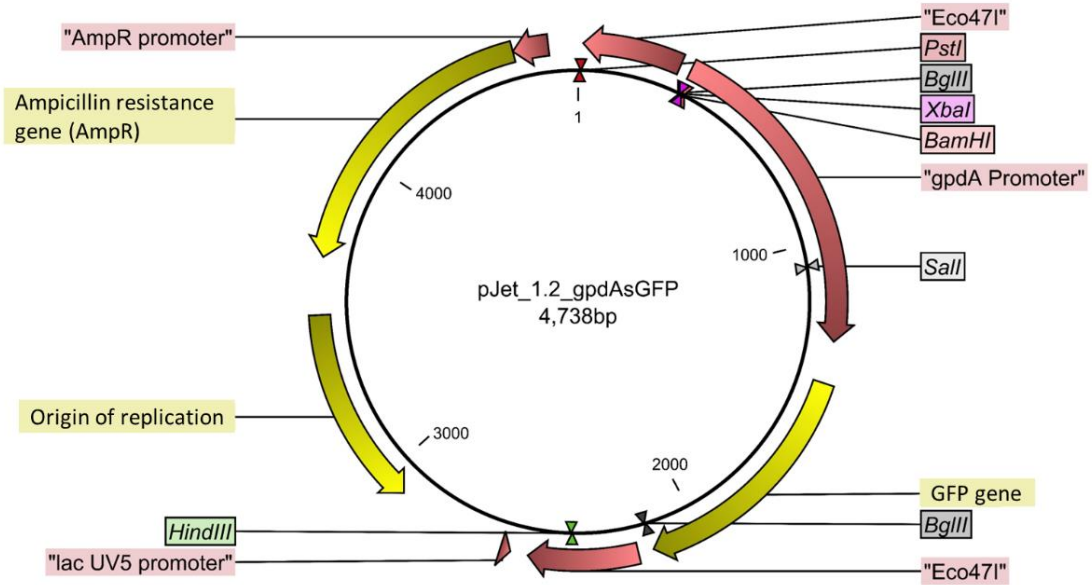

Figure S2: pJET_1.2_gdpAsGFP plasmid map. The *GFP* gene was cloned into the pJET1.2/blunt vector (Marais, 2020). This is a positive selection cloning vector which contains a gene that expresses a lethal restriction enzyme, Eco471. This gene is under control of the lac UV5 promoter, a mutated lac operon promoter that is constitutively active and promotes high levels of transcriptional expression in *Escherichia coli*. The vector cloning site is contained within the *Eco471* sequence and successful ligation of the insert sequence into the cloning site disrupts the expression of the lethal gene. The expression of the *GFP* gene is initiated by a constitutive promoter, gdpA. This promoter sequence was cloned from *Aspergillus nidulans* and is responsible for the expression of the *gpdA* gene which encodes glyceraldehyde-3-phosphate dehydrogenase (GPD).

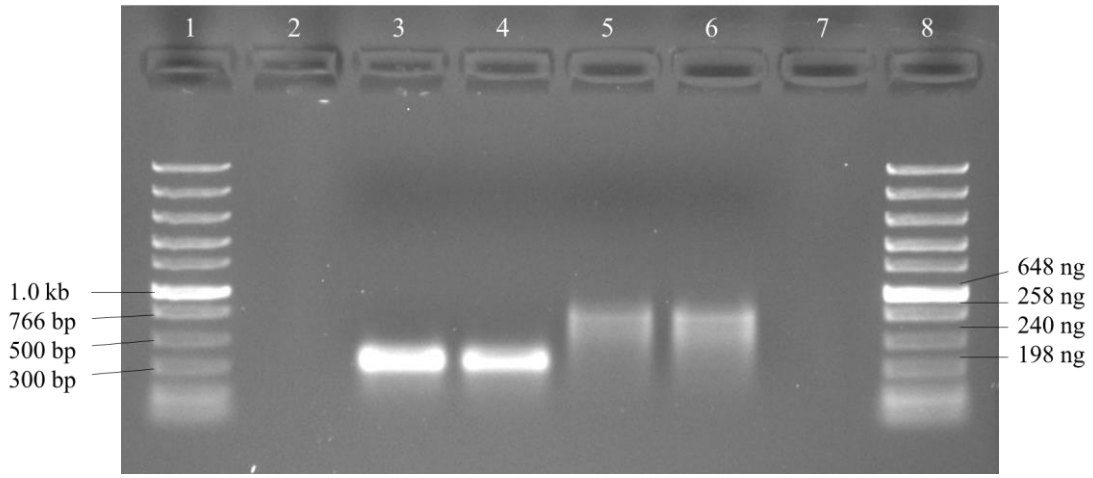

Figure S3: GFP dsRNA and 3Cz dsRNA products subjected to gel electrophoresis. Lanes 1 and 8 contain the 1 kb Fast DNA ladder (NEB) and the added notations indicate the ladder's expected band sizes (left) and amount of DNA in ng (right). Lane 2 is empty. Lanes 3 and 4 contain the GFP dsRNA which yielded the expected size of 365 bp. Lanes 5 and 6 contain the 3Cz dsRNA which yielded a size of approximately 766 bp instead of the full length of 1.4 kb (see results Section 3.1 for more details). Lane 7 is empty. 98-100 ng of dsRNA samples were added per well. The products were separated on a 1.2% agarose gel made with TAE buffer and stained with EtBr.

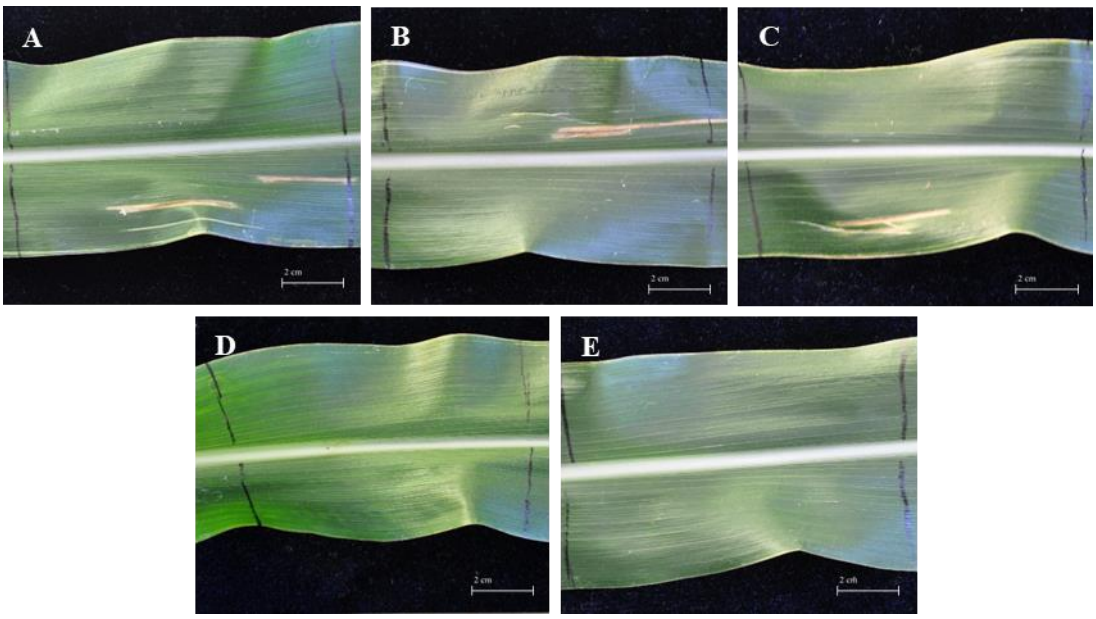

Figure S4: Representative images of GLS disease symptoms recorded 38 dpi for various treatments in the second SIGS trial. Treatments included: **A)** *C. zeina* (*Cz*, positive disease control), **B)** *Cz* + GFP dsRNA (dsRNA specificity negative control), **C)** *Cz* + GFP dsRNA applied 1 dpi, **D)** *Cz* + 3Cz dsRNA (test construct targeted towards three pathogenicity genes of *C. zeina*), **E)** *Cz* + 3Cz dsRNA applied 1 dpi. The most severe GLS disease scores were observed in the *C. zeina*-inoculated leaves (positive disease control) and inoculated leaves which received the GFP dsRNA treatments.


C. berteroae MPGLARKLLI FAAVDGLIIQ SA---GQRGS GGTRIAYGTN NITAASEKDR EPEGGG--SL EAHGLVGVLN LVSSSYLIAI TRRSEVANIR ERPIFRITDV TLIPLASRGE ASNAISHAEK LLHQNSSGAE QDTDESDVGE DVETGSVVTD 145
C. beticola MPGLARKLLI FAAVDGLIIQ SA---GQRGS GGTRMEYGTN NITAASEKDR GPEGGG--SL EAHGLVGVLN LVSSSYLIAI TRRSEVANIR ERPVFRITDV TLIPLASRGE ASNAISHAEQ LLHQNSSGAE QDTDESDVGE DVETGSVVTD 145
C. kikuchii MPGLARKLLI FAAVDGLIIQ SA---GQRGS GATRIEYGTN NITAASEKDR GPEGGG--SL EAHGLVGVLN LVSSSYLIAI TRRSEVANIR ERPVFRITDV TLIPLASRGE ASNAISHAEK LLHQNSPGAE QDTDESDVGE DVETGSVGTD 145
C. zeae-maydis MPGLARQLLI FAAVDGLIVQ SA---GHRGG GATRIEYGTN NITAVSEKDR QPEGSD--LL EAHGLI---- ---------- -----VAQWR EKPVFRVTDV TLIPLASRAE AASAIAHAER LLHQKTSAAE HDTEDSDVGE EVETGSVGTD 126
C. zeina MPGLARKLLI FAAVDGLIVH SA---GQRGG RGTRIEYGTN TITAVSEKDH QPERSG--LL EAHGLVGLLN LVSSSYLIAI TRRSEVAQWR EKPVFRITDV TLIPLASRAE ASSAIAHAER LLHQNTSWAE HDTDESDVGE EVETGSVGTD 145
B. cinerea MPGLVRKLLI FAAIDGLILQ PAAPKGQRPA PATKIAYKDK HIGPVLSDLQ DLEGSSAKSF EAFGIVGLLT VSKSSFLISI TKREQVAQIQ GKPIYVITEV ALTPLSSKNE AEISIDSTKA GLLKSNIEGQ HGLDESDSED DVVSDEV--- 147
Consensus MPGLARKLLI FAAVDGLIIQ SA---GQRGS GXTRIEYGTN NITAXSEKDR XPEGXG--SL EAHGLVGVLN LVSSSYLIAI TRRSEVAXIR EXPVFRITDV TLIPLASRGE ASNAISHAEX LLHQNSSGAE XDTDESDVGE DVETGSVGTD
100%
Conservation
0%
160 180 200 220 240 260 280 300
C. berteroae SGENVPPRNP EDESTALEAP KPGTIKQSTT FVKDVVQQKG TFGRFATSWF SKKGTQTDSQ RKSDAEPDQK LNMEQKKQEM GALPDTDEDK SEEAGEAQAE PKGRS----S TEKQRQSAID YLAPRIVKSA RLYFSTS-GF YFSYDHDLSH 290
C. beticola SGENVPARNP EDESTALEAP KPGTIKQSTT FVKDVVQQKG TFGRFATSWF SKKGTQTDSQ PKSDAEPDQK LNMEQKKQEM GALPDTDKDQ SEKANEAQEE PKGRS----S TEKRRQSAID YLALRIVKSA RLYFSTS-GF YFSYDHDLSH 290
C. kikuchii SGENAPARNP EDDSTALEAP KPGTIKQSTT FVKDVVQQKG TFGRFATSWF SKKGTQTDSQ RKSDAEPDQK LNMEQKKQEM EALPDTDKNQ TEEAGEAQEE PKGRS----S TEKRRQSAID YLAPRIVKSA RLYFSTS-GF YFSYDHDLSH 290
C. zeae-maydis AGDNVPEQNP EEESTALEAG KPGIIKQRTT IVKDVVQQKG TFGRFATSWF SKRGTQSNSQ RQSHAESGES -NKEQEKHEA EPLPDADNSQ AAEIGKLQEE PKGMS----S TERRRQSAID YLAPRIVKSA RLYFSTS-GF YFSYDHDLSR 270
C. zeina AGDNIPERNL EDESAALEPP QPGTIKQSTT FVKDVVQQKG TFGRFATSWF RKRGAQGNSQ RQSDAESKAS -NKEDEKQDA QVSPDADSSQ AAEIRKSQEE PKGRS----S TERRRQSAID YLAPRIVESA RLYFSTS-GF YFSYDHDLSQ 289
B. cinerea ---------- -EDDTAVEAH -----KRTSS VAEDVISKKG GYGRFAQKWF SKKGWAVD-Q KKNLGMSAEP YSTVEQASKA TDVPATISGV TEGKSDISIP DKGKEIEDIE TPENISDIAE SMLPKLLRTS QILFGASRSY YFSYDHDITR 280
Consensus SGENVPXRNP EDESTALEAP KPGTIKQSTT FVKDVVQQKG TFGRFATSWF SKKGTQTDSQ RKSDAEXDQK LNMEQKKQEX XALPDTDXXQ XEEAGEAQEE PKGRS----S TEKRRQSAID YLAPRIVKSA RLYFSTS-GF YFSYDHDLSH
100%
Conservation
0%
320 340 360 380 400 420 440
C. berteroae SLGRDAPSSS AVPLWKRFDS LFFWNRHLCS PLAAAGQDSL VLPLIQGFVG QRAFSI-AQA DYRAIAAEPG DT-----ELT DFNERE---A SKNAQQHNFH VTLISRRSVK RAGLRYLRRG VDDQGNVANS VETEQILSTQ VDDSKQKIFS 431
C. beticola SLGRKATSSS AVPLWKRFDS LFFWNRHLCS PLADAGQDSL VLPLIQGFVG QRAFSI-AQA DDRAIVAEPG DT-----ELT DFNERE---A SKNAQQHNFH VTLISRRSVK RAGLRYLRRG VDDQGNVANS VETEQILSTQ VDDSKQKIFS 431
C. kikuchii SLGRNATSSS AVPLWKRFDS LFFWNRHLCS PLADAGQDSL VLPLIQGFVG QRAFSI-AQA DDRAIVAEPD DT-----ELT DFNERE---A SKNAQQHNFH VTLISRRSVK RAGLRYLRRG VDDQGNVANS VETEQILSTQ VDDAKQKIFS 431
C. zeae-maydis SLGRDVPSSS TLPLWKRSDD LFFWNWHLCS PLANAGQDSL VLALIQGFVG QRAFSV-TPA DDQVIVAEPN DT-----ELT DFNEDE---A SREPEKHNFH VTLISRRSVK RAGLRYLRRG VDDQGNVANS VETEQILSTQ VNDSARKTFS 411
C. zeina SLGKDVLSSS TPPLWKRFDS IFFWNWHLCS PLANAGQDSL VLPLIQGFIG QRAFSI-TQA DDHVVVAEPD DT-----ELT DFNEGE---S NKKSEKHDFH VTLISRRSVK RAGLRYLRRG VDDQGNVANS VETEQILSTQ VHDSTQKTFS 430
B. cinerea SLANKRNTNS ELPLHKEVDP LFFWNRHLTL PFIDAGQSSL ALPLMQGFVG QRAFSMDSNP PNPAIGSDTG KTSVQMKDIT TSSSDEQIYT ARAGTDKSYL LTLISRRSVK RAGLRYLRRG VDEDGNTANG VETEQILSDS AWGPSSKTYS 430
Consensus SLGRDAXSSS AVPLWKRFDS LFFWNRHLCS PLADAGQDSL VLPLIQGFVG QRAFSI-AQA DDRAIVAEPG DT-----ELT DFNERE---A SKNAQQHNFH VTLISRRSVK RAGLRYLRRG VDDQGNVANS VETEQILSTQ VDDSKQKXFS
100%
Conservation
0%
460 480 500 520 540 560 580 600
C. berteroae LLQYRGSMPL FFSQSPYSFK PTPIMFGSEA TNQAAFQKHF KSLAARYGDI QAASLIDKHG TELNIGTAYE EAAKKLNESD GID-GKPLSF HWFDFHGECK GMRFDKVNLL IDSLKLFLKS SAFTVEQDGE LKQKQSGILR TNCMDCLDRT 580
C. beticola LLQYRGSMPL FFSQSPYSFK PTPIMFGSEA TNQAAFQKHF KSLAARYGDI QAASLIDKHG TELNIGTAYE EAAKKLNESD GID-GKPLGF QWFDFHGECK GMRFDKVNLL IDSLKSFLKS SAFTVEQDGE LKQKQSGILR TNCMDCLDRT 580
C. kikuchii LLQYRGSMPL FFSQSPYSFK PTPIMFGSEA TNQAAFQKHF KSLAARYGDI QAASLIDKHG TELNIGTAYE QAAKKLNESN GID-GKPLGF QWFDFHGECK GMRFDKVNLL IDSLKSFLES SAFTVEQDGE LKQKQSGILR TNCMDCLDRT 580
C. zeae-maydis LLQYRGSMPL FFSQSPYSFK PTAIMFGSES TNQAAFQKHF KNLATRYGDV QAASLIDKRG TELNIGVAYE EAAKKLNENG GID-GKPLGF QWFDFHGECK GMRFDKVNLL VNSLKSFLTS SAFTVEQGGA IKQKQTGILR TNCMDCLDRT 560
C. zeina LLQHRGSMPL FFSQSPYSFK PTPTMFGSES INQAAFQKHF KNLAARYGDV QAASLIDKHG TELNIGVAYE EAAKKLNENG GID-GKPLGF RWFDFHGECK GMRFDKVNLL INSLKSILTS SAFTVEQDGA IKQKQTGILR TNCMDCLDRT 579
B. cinerea FVQIRGSIPI FFSQSPYSFK PVPQVHHSTE TNYEAFKKHF DNISDRYGAI QVASLVEKHG NEAIVGGEYE KLMTLLNVSR ASELRKSIGF EWFDFHAICK GMKFENVSLL MEILDKKLDS FSHTVETDGK LVSKQNGVLR TNCMDCLDRT 580
Consensus LLQYRGSMPL FFSQSPYSFK PTPIMFGSEA TNQAAFQKHF KXLAARYGDI QAASLIDKHG TELNIGTAYE EAAKKLNESX GID-GKPLGF QWFDFHGECK GMRFDKVNLL IDSLKSFLXS SAFTVEQDGE LKQKQSGILR TNCMDCLDRT
100%
Conservation
0%
620 640 660 680 700 720 740

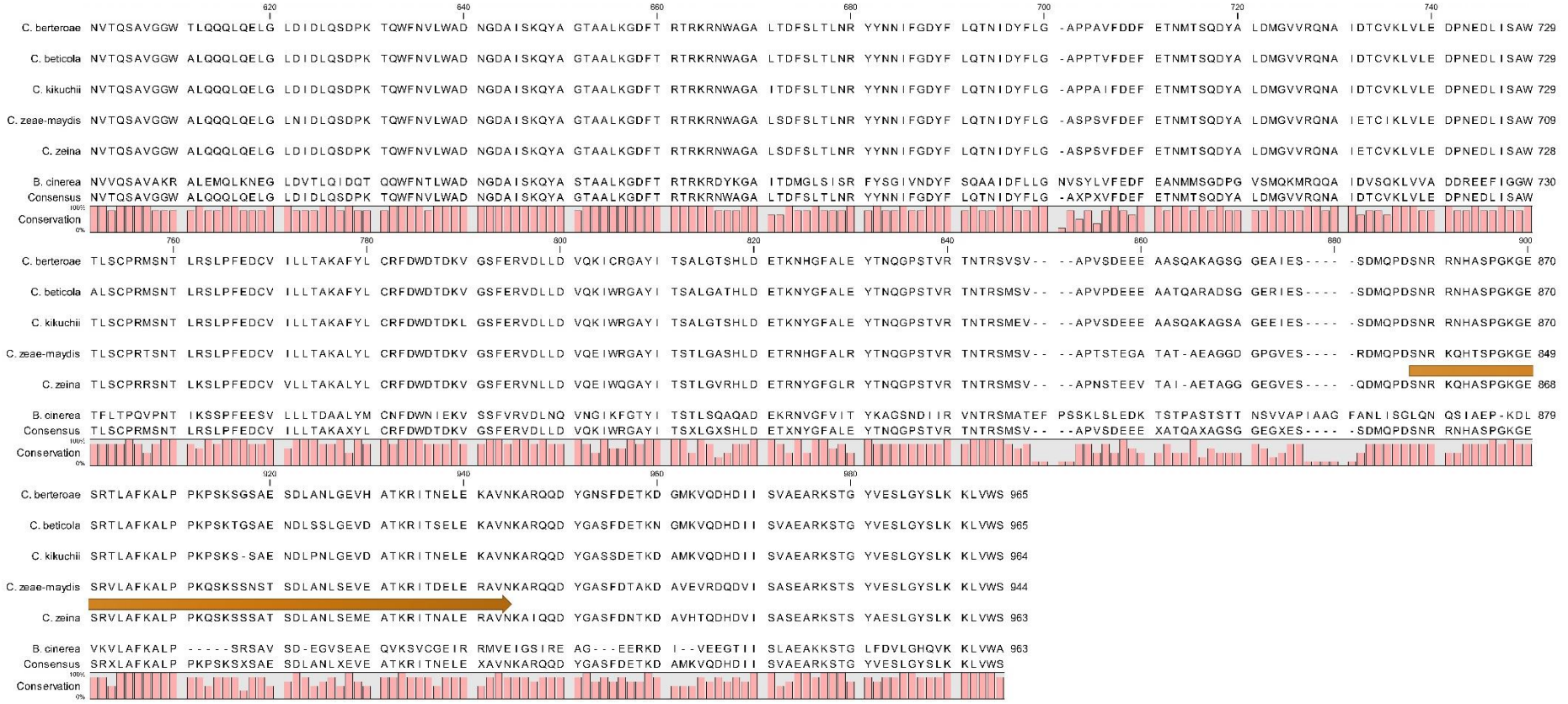

Figure S5: Suppressor of actin 1 (SAC1)-like phosphoinositide phosphatase enzyme amino acid sequence alignments between *Botrytis cinerea* and *Cercospora* species. The orange annotation indicates the section which the SAC1/ VPS51/DCTN1 dsRNA construct targets, which was designed in a highly conserved section towards the 3'-end of the gene. Percentage sequence conservation between different species is indicated with the bar chart in pink.


C. berteroae MDGAKAKASK EAATQDIKSQ NKKRQLEFRA QHGLVVGFFL LIL---IFHA LGIYLFLNGF LLSRLVLQHR SECANPPIDL TGHPKDFAP- --GSQEQGCW HPKSFDK-AV VIIVDALRYD FTTPFQPKPG AESPQHFHDA LPVLYETSVN 143
C. beticola MDGAKAKASK EAATQDIKAQ NKKRQLEFRA QHGLVVAFFL LIL---IFHA LGIYLFLNGF LLSRLVLQHR SECAHPPINL TGHSKDFTP- --GSQEQGCW HPKSFDK-AV VIIVDALRYD FTTPFQPKAG AETPQHFHDA LPVLYETSVN 143
C. kikuchii MDGAKAKASK EAATQDIKAQ NKKRQLEFRA QHGLVVAFFL LIL---VFHA LGIYLFLNGF LLSRLVLQHR SECAHPPIDL TGHPKDFTP- --GSQEQGCW HPKSFDK-AV VIIVDALRYD FTTPFQPKPG AESPQHFHDA LPVLYETSVN 143
C. zeae-maydis MDTATAKVSK ETATQDIKAQ NKKRQLEFRA QHGLVVAFFL LILHHRIFHA LSVYLFLNGF LLSRLVLQHR SECAKPPIDL VGHPRGFTP- --GSQEQGCW HPKSFDK-AV VILVDALRYD FTIPFQSKPG HETSQHFHDA LPVLYETSVK 146
C. zeina QK-------- ---------- ---------- ---------- ----HRIFHA LGIYLFLNGF LLSRLVLQHR SECANPPVDL ARHPKDFTP- --GSQEQGCW HPKSFDK-AV VIIVDALRYD FTIPFQSKPG HETPHHFHDA LPVLYETSVD 104
B. cinerea MPG------- ---------- ---------- ---------- ------LVRK LLIFAAIDGL ILQPAA--PK GQRPAPATKI AYKDKHIGPV LSDLQDLEGS SAKSFEAFGI VGLLTVSKSS FLISITKR-- EQVAQIQGKP IYVITEVALT 103
Consensus MDGAKAKASK EAATQDIKAQ NKKRQLEFRA QHGLVVAFFL LIL---IFHA LGIYLFLNGF LLSRLVLQHR SECAXPPIDL TGHPKDFTP- --GSQEQGCW HPKSFDK-AV VIIVDALRYD FTXPFQPKPG AETPQHFHDA LPVLYETSVN
Conservation
C. berteroae EPQNAFLRPF IADPPTTTLQ RLKGLTTGTL PV-LIDAGSN FAGTAIDEDN IVEMLYKAGK KVVHLGDDTW HSLFPGYFEP NLTKAYDSFN VWDLHTVDN- GVNEHLFPLL EPSMSGRWDV IFGHYLGVDH AGHRYGPDHP AMNDKLKQMD 291
C. beticola EPQNAFLRPF IADPPTTTLQ RLKGLTTGTL PV-LIDAGSN FAGTAIDEDN IVEMLYKAGK KVVHLGDDTW HSLFPGYFEP NLTKAYDSFN VWDLHTVDN- GVNEHIFPLL DPSMSGRWDV IFGHYLGVDH AGHRYGPDHP AMNDKLKQMD 291
C. kikuchii EPQNAFLRPF IADPPTTTLQ RLKGLTTGTL PV-LIDAGSN FAGTAIDEDN IVEMLYKAGK KVVHLGDDTW HSLFPGYFEP NLTEAYDSFN VWDLHTVDN- GVNEHIFPLL DPSMSGRWDV IFGHYLGVDH AGHRYGPDHP AMNDKLKQMD 291
C. zeae-maydis EPQNAFLRPF IADPPTTTLQ RLKGLTTGTL PV-LIDAGSN FAGTAIDEDN IVEMLYNAGK KVVHLGDDTW HSLFPGHFEP NLTKAYDSFN VWDLHTVDN- GVNEHLFPLL EPSMSGRWDV IIGHYLGVDH AGHRYGPDHP AMNDKLKQMD 294
C. zeina EPQNAFLRPF IADPPTTTLQ RLKGLTTGTL PV-LIDAGSN FAGTAIDEDN IVEMLYKAGK KVVHLGDDTW HSLFPGHFEP NLTKAYDSFN VWDLHTVDN- GVNEHLFPLL EPSMAGRWDV IFGHYLGVDH AGHRYGPDHP AMNEKLKQMD 252
B. cinerea PLSSKNEAEI SID--STKAG LLKSNIEGQH GLDESDSEDD VVSDEVEDDT AVE----AHK RTSSVAEDVI -SKKGGY--G RFAQKWFSKK GWAVDQKKNL GMSAEPYSTV EQA-SKATDV ------PATI SGVTEGKSDI SIPDKGKEIE 237
Consensus EPQNAFLRPF IADPPTTTLQ RLKGLTTGTL PV-LIDAGSN FAGTAIDEDN IVEMLYKAGK KVVHLGDDTW HSLFPGYFEP NLTKAYDSFN VWDLHTVDN- GVNEHLFPLL EPSMSGRWDV IFGHYLGVDH AGHRYGPDHP AMNDKLKQMD
Conservation
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C. beticola DV-IRRIVAA LDDQTL--LV VMGDHGMDVK GDHGGESDDE VQAALWMYSK RGIFGRKDKD SLLPPPTAKE RPVAQIDLVP TLSLLLGLPV PFNNLGH--- --PIEEAFHG SSTL-LKPNY DNLAQVSRLT AAQIHKYQAE YAKARGLGNS 432
C. kikuchii DV-IRHMVAA LDDQTL--LV VMGDHGMDVK GDHGGESDDE VQAALWMYSK RGIFGRKNKD SLLPPLTAKE RPVAQIDLVP TLSLLLGLPV PFNNLGH--- --PIEEAFHG SSSL-LKPNY DNLAQVSRLT SAQIHKYQAE YAKARGLGNS 432
C. zeae-maydis GV-IRRMVAR LDDKTL--LV VMGDHGMDVK GDHGGESDDE VQAALWMYSK RGIFGRQDKE SLLPPLNAKE RPVAQIDLVP TLSLLLGLPV PFNNLGH--- --PIEEAFHG SSTL-LQPNY DNLAQVSRLT AAQIHIYQAE YAKARGLGNS 435
C. zeina DV-IRRMVAG LDDKTL--LV VMGDHGMDVK GDHGGESDDE VQAALWMYSK RGIFGRKDKE SLLPPLTAKE RPVAQIDLVP TLSLLLGLPV PFNNLGH--- --PIEEAFHG SSTL-LKPNY ENLAQVSRLT AAQIHIYQAE YAKARGLGNS 393
B. cinerea DIETPENISD IAESMLPKLL RTSQILFGAS RSYYFSYDHD IT-------- RSLANKRNTN SELP--LHKE -----VD--P LFFWNRHLTL PFIDAGQSSL ALPLMQGFVG QRAFSMDSNP PNPAIGSDTG KTSVQMKDIT TSSSDEQIYT 370
Consensus DV-IRRMVAA LDDQTL--LV VMGDHGMDVK GDHGGESDDE VQAALWMYSK RGIFGRKDKD SLLPPLTAKE RPVAQIDLVP TLSLLLGLPV PFNNLGH--- --PIEEAFHG SSTL-LKPNY DNLAQVSRLT AAQIHKYQAE YAKARGLGNS
Conservation
C. berteroae TSSTTDQLWK LANQSWGSKE RT-------- --EAWGAFLA YQTENLRICK SLWARFDLVS MIMGIIALIG TFLVVLVYA- QGIVRGDRAA MTPPLFTWGT IGAVGGAAAG AAVGFLTTEH AVQSIAFGAV FGGVTATLAM FRPALELLRI 571
C. beticola TSSTTDQFWK LANQSWGSKE RT-------- --EAWDAFLA YQTENLRICK SLWARFDLTS MTMGIIALIG TFLVVVIYA- QGIVRGDRAA MTPPLFTWGT IGAVVGAAAG AAVGSLTTEH GVQSIAFGAV FGGVTATLAM FRPALELLRI 571
C. kikuchii TSSTTDQLWK LANQSWGSKE RT-------- --EAWDAFLA YQTENLRICK SLWARFDLTS MTMGIIALIG TFLVVVIYA- QGIVRGDRAA MTPPLFTWGT IGAVVGAAAG AAVGSLTTEH GVQSIAFGAV FGGVTATLAM FRPALELLRI 571
C. zeae-maydis TSSTTDEFWR LANQAWSSSE RK-------- --ESWNAFLA YQTENLRICK SLWARFDLVS MAMGIVALIG TFLVVLVYA- QGIVRGDRAA LTPPLFTWGT IGAVGGAAAG AAVGSLTAEH AVQSIAFGAV FGGVTTTLAM FRPALELLQI 574
C. zeina TSSTTDHLWR LANQAWSSSE RE-------- --ESWNAFLA YQTENLRICK SLWARFDLVS MTMGIIALIG TFLVVLVYA- QGIVRGDRAA MTPPLFTWGT IGAVGGAAVG AAVGSLTTEH AVQSIAFGAV LGGVSATLTM FRPALELLRI 532
B. cinerea ARAGTDKSYL LTLISRRSVK RAGLRYLRRG VDEDGNTANG VETEQI-LSD SAWGPSSKTY SFVQIRGSIP IFFSQSPYSF KPVPQVHHST ETNYEAFKKH FDNISDRYGA IQVASLVEKH GNEAIV-GGE YEKLMTLLNV SR-ASEL--- 514
Consensus TSSTTDQLWK LANQSWGSKE RT-------- --EAWNAFLA YQTENLRICK SLWARFDLXS MTMGIIALIG TFLVVLVYA- QGIVRGDRAA MTPPLFTWGT IGAVGGAAAG AAVGSLTTEH XVQSIAFGAV FGGVTATLAM FRPALELLRI
Conservation
C. berteroae PRPTTFWGWL CTLSTLMLCG GFA-ANSFII WE--DEQLLF LLTTFA---- -VAMLGSSLG QGDPDDRWLG ATNAVSFLIA TRLSSFSRLC RE-------- EQMPFCRSTY YASATSSTSS KWQLAIPLSV ALILPSAITH FYKRSANYHG 705
C. beticola PRPTTFWGWL CTLSTLMLCG GFA-ANSFII WE--DEQLLF LLTTFA---- -VAMLGSSLG QGDPDDRWLG ATNAVSFLVA TRLSSFSRLC RE-------- EQMPFCRSTY YASATSSTSS KWQLAIPLSV ALILPSAITH FYKRSANYHG 705
C. kikuchii PRPTTFWGWL CTLSTLMLCG GFA-ANSFII WE--DEQLLF LLTTFA---- -VAMLGSSLG QGDPDDRWLG ATNAASFLIA TRLSSFSRLC RE-------- EQMPFCRSTY YASATSSTSS KWQLAIPLSV ALILPSAITH FYKRSANYHG 705
C. zeae-maydis PRPTTFWGWL CTLSTVLLCG GFA-ANSFII WE--DEQLLF LLTTFA---- -VAMLGSSLG QGDPDDRWLG ATNAVSFLVA TRLSSFSRLC RE-------- EQMPFCRSTY YASATSSTSS KWQLAIPLSV ALILPSAITH FYKRSANYHG 708
C. zeina PRPTTFWGWL CTLSTVLLCV GFA-ANSFII WE--DEQLLF LLTTFA---- -VAMLGSSLG QGDPDDRWLG ATNAASFLIA TRLSSFSRLC RE-------- EQMPFCRSTY YASATSSTSS KWQLAIPLSV ALILPSAITH FYKRSANYHG 666
B. cinerea -RKSIGFEWF ---DFHAICK GMKFENVSLL MEILDKKLDS FSHTVETDGK LVSKQNGVLR TNCMD--CLD RTNVVQSAVA KRALEM-QLK NEGLDVTLQI DQTQQWFNTL WADNGDAISK QYA-----ST AA-LKGDFTR TRKR--DYKG 649
Consensus PRPTTFWGWL CTLSTLMLCG GFA-ANSFII WE--DEQLLF LLTTFA---- -VAMLGSSLG QGDPDDRWLG ATNAVSFLXA TRLSSFSRLC RE-------- EQMPFCRSTY YASATSSTSS KWQLAIPLSV ALILPSAITH FYKRSANYHG
Conservation

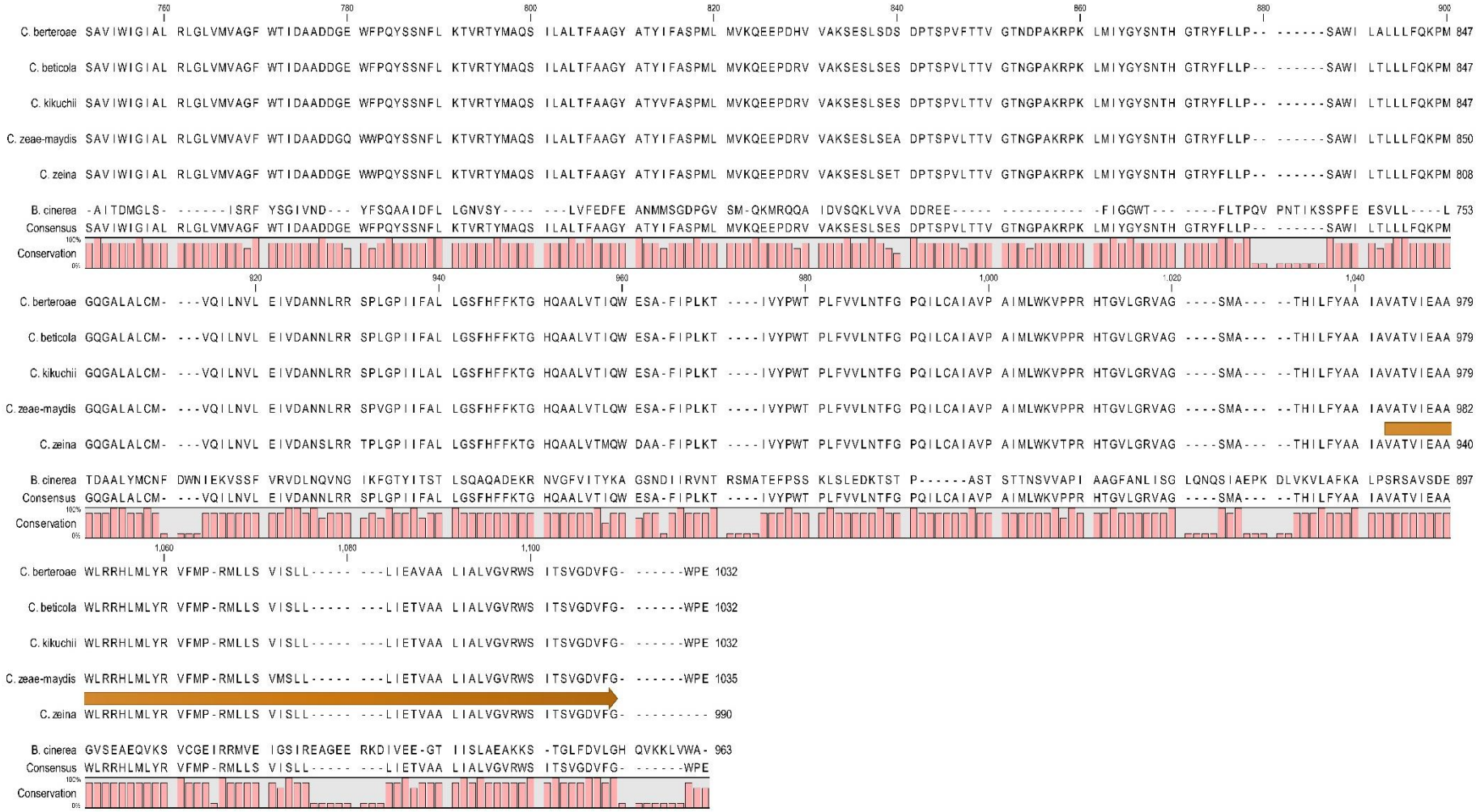

Figure S6: Vacuolar protein sorting 51 (VPS51) amino acid sequence alignments between *Botrytis cinerea* and *Cercospora* species. The orange annotation indicates the section which the SAC1/ VPS51/DCTN1 dsRNA construct targets, which was designed in a highly conserved section towards the 3'-end of the gene. Percentage sequence conservation between different species is indicated with the bar chart in pink.

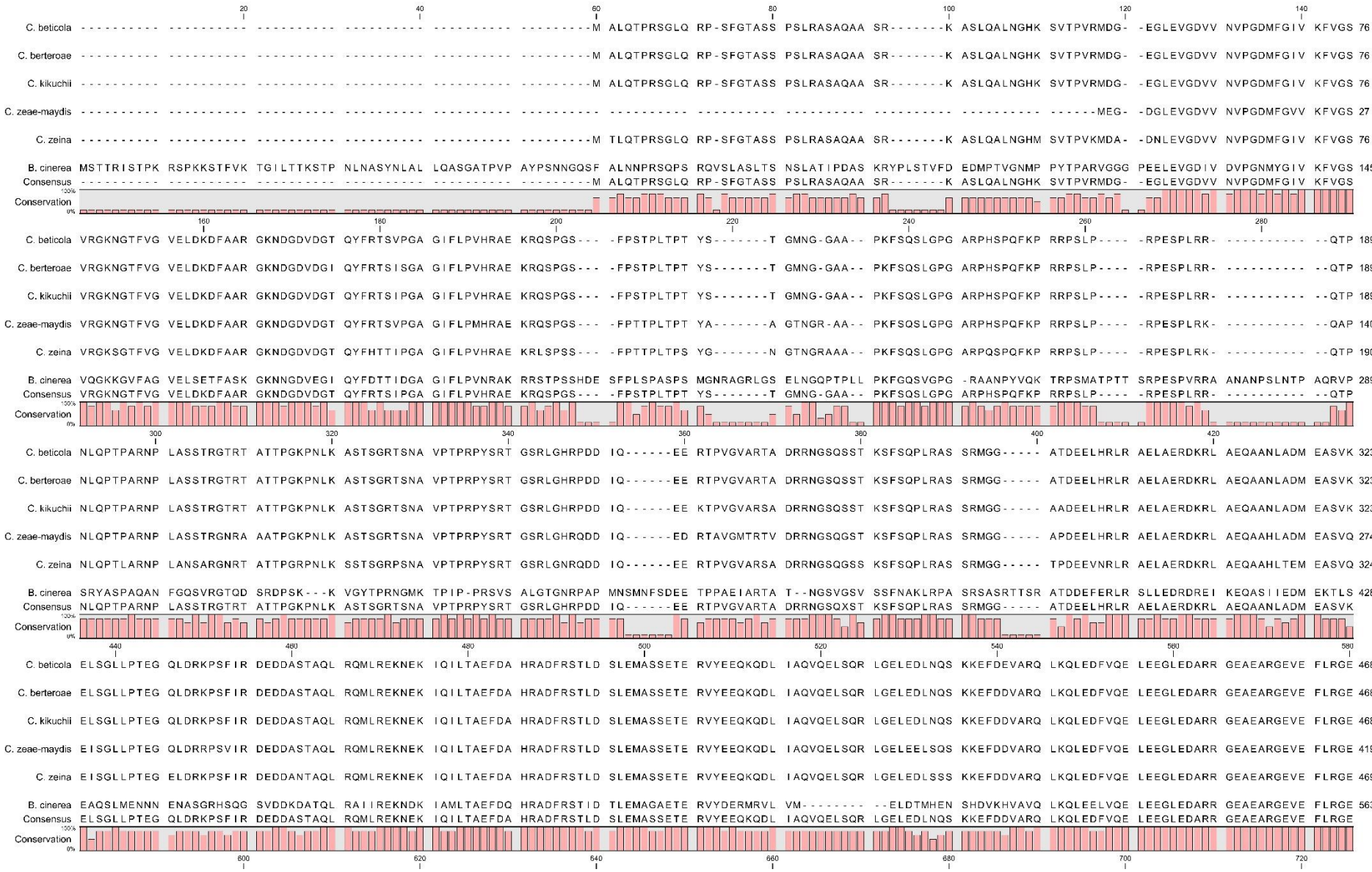
C. beticola
C. berteroae
C. kikuchii
C. zeae-maydis
C. zeina
B. cinerea
Consensus
Conservation

Figure S7: Dynactin (DCTN1) amino acid sequence alignments between *Botrytis cinerea* and *Cercospora* species. The orange annotation indicates the section which the SAC1/ VPS51/DCTN1 dsRNA construct targets, which was designed in a highly conserved section towards the 3'-end of the gene. Percentage sequence conservation between different species is indicated with the bar chart in pink.

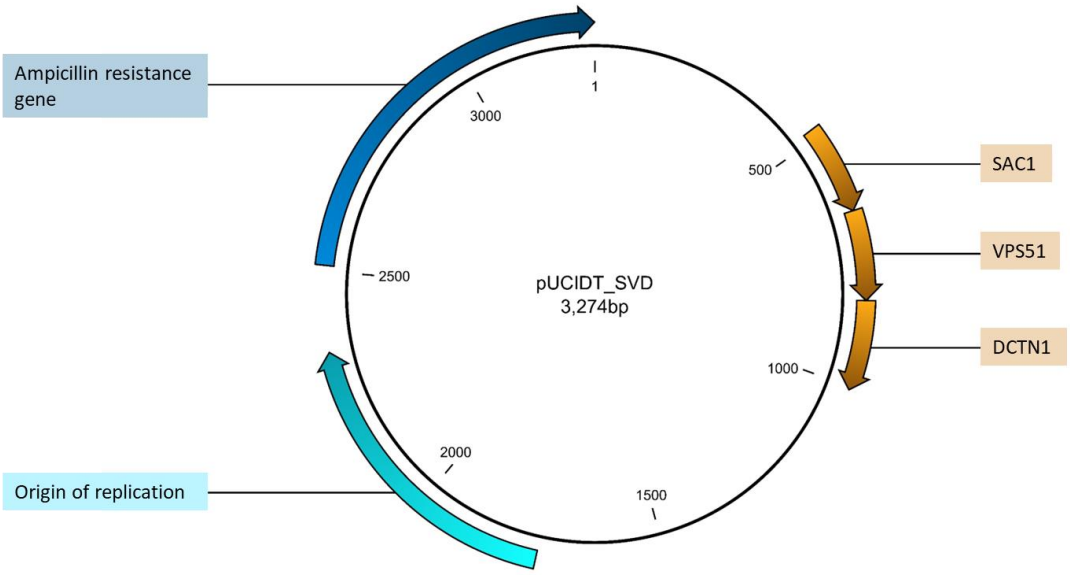

Figure S8: pUCIDT_SVD plasmid map. The *SAC1/VPS51/DCTN1* sequence was synthesised and cloned into the pUCIDT cloning vector.

Supplementary Tables

Table S1: The total area under disease progress curve (AUDPC) scores recorded. Total AUDPC scores were calculated at the end of the first and the second trials for the *C. zeina* disease control, GFP dsRNA specificity negative control, and 3Cz dsRNA test construct treatments, applied immediately and one day post inoculation (dpi).

	Replicate	*C. zeina* (Cz)	Cz + GFP dsRNA	Cz + GFP dsRNA (1 dpi)	Cz + 3Cz dsRNA	Cz + 3Cz dsRNA (1 dpi)
First trial	1	42	27	93	66	18
	2	36	57	30	48	18
	3	24	45	27	42	21
	4	30	57	33	42	18
	5	45	N/A	N/A	N/A	N/A
	6	78	N/A	N/A	N/A	N/A
	Mean	**42.5**	**46.5**	**45.75**	**49.5**	**18.75**
Second trial	1	78	66	90	30	45
	2	72	51	78	63	45
	3	96	66	48	93	48
	4	66	66	78	45	45
	5	72	N/A	N/A	N/A	N/A
	6	66	N/A	N/A	N/A	N/A
	Mean	**75**	**62.25**	**73.5**	**57.75**	**45.75**

Table S2: The germination efficiency of *C. zeina* conidia grown *in vitro*. The mean percentage of germinated conidia growing in water and on V8 agar culture media was measured over four intervals in 24 h.

Time	**0 h**	**3 h**	**7 h**	**16 h**	**24 h**
Water (% germinated conidia)	6.53	23.3	30.6	33.2	33.7
V8 Agar Media (% germinated conidia)	6.53	58.0	62.5	72.4	78.7

Table S3: The germination efficiency of *C. zeina* conidia grown *in planta*. The conidial germination percentage was determined from observations made on scanning electron microscopy (SEM) images of plant leaf samples inoculated with a *C. zeina* conidial suspension. Inoculated leaf samples were collected at four intervals over a period of four days. Intervals are measured in hours post inoculation (hpi) and days post inoculation (dpi).

Time	**0 hpi**	**16 hpi**	**24 hpi**	**4 dpi**
Number of SEM images	8	10	60	45
Number of biological replicates	2	4	6	2
Number of germinated conidia	12	24	71	34
Number of conidia	28	34	92	44
% Germinated conidia	42.8	70.6	77.2	77.3