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ORIGINAL ARTICLE

Double-stranded RNA uptake for the control of the maize pathogen *Cercospora zeina*

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Abstract

RNA interference (RNAi) using double-stranded RNA (dsRNA) against fungal pathogens is an emerging field of crop disease control. We aimed to evaluate RNAi against the fungus *Cercospora zeina* causing grey leaf spot (GLS) disease on maize. Orthologues of *Dicer-like 1*, *Dicer-like 2*, *RNA-dependent RNA polymerase* and two copies of *Argonaute* were identified in the *C. zeina* genome and were shown to be expressed in vitro and in planta. Confocal microscopy showed that *C. zeina* took up exogenously applied dsRNA labelled with fluorescein. GFP-transgenic *C. zeina* was treated with GFP-specific dsRNA, and GFP mRNA expression and protein fluorescence were reduced by 57% and 61%, respectively. A Cz3-dsRNA targeting *C. zeina chitin synthase D* (*CHSD*), *phosphatidylserine decarboxylase proenzyme 3* (*PSD3*) and *extracellular protein 2* (*ECP2*) was constructed. Treatment of *C. zeina* cultures with the Cz3-dsRNA reduced *CHSD* expression by 47% and reduced cell viability by 34%. Maize leaves were inoculated with *C. zeina* conidia, and Cz3-dsRNA was applied either with the conidia or 16 h later. GLS disease was significantly reduced compared to the water control for the 16 h post-inoculation (hpi) treatment with Cz3-dsRNA, but not for the GFP-dsRNA specificity control or treatments at 0 hpi. We hypothesized that germination of *C. zeina* conidia was required for effective dsRNA-mediated control, and this was borne out by microscopy observations that most of the *C. zeina* conidia (70%) germinated successfully on the maize leaf surface within 16 hpi. This work lays the groundwork for a dsRNA-based fungicide against this foliar pathogen.

KEYWORDS
chitin synthase, grey leaf spot disease, RNAi, SIGS, spray-induced gene silencing

1 | INTRODUCTION

The production of maize, a staple food crop globally, is threatened by a variety of pests and diseases, one of them being the foliar disease grey leaf spot (GLS), caused by the fungal pathogen *Cercospora zeina* (Crous et al., 2006; Wang et al., 1998). *C. zeina* belongs to the *Dothideomycete* class, which contains a variety of well-studied plant-pathogenic fungi (Haridas et al., 2020). GLS causes necrotic lesions on the maize leaf blade leading to major yield losses of up to 65% in susceptible varieties (Meisel et al., 2009;

Ward et al., 1999). *C. zeina* spores overwinter within the soil and when favourable environmental conditions such as relative high temperatures together with high humidity occur, they will germinate and start to infect maize plants (Meisel et al., 2009; Ward et al., 1999).

The closely related GLS pathogen *Cercospora zeae-maydis* penetrates maize leaves by stomatal tropism, formation of appressoria and penetration through stomata (Beckman & Payne, 1982). A similar mode of stomatal penetration by *C. zeina* is likely as the accumulation of hyphae in the substomatal cavity was shown in a microscopy study from Cedara in South Africa (Caldwell & Laing, 2005). The pathogen was named *C. zeae-maydis* in this report; however, it was prior to the naming of *C. zeina* (Crous et al., 2006). We first sampled GLS-infected maize at Cedara in 2011 (Muller et al., 2016). All 54 isolates were *C. zeina* and none were *C. zeae-maydis* (Muller et al., 2016), which appears to be absent in sub-Saharan Africa (Nsibo et al., 2021).

Fungicides are an important management strategy for GLS disease of maize (Mallowa et al., 2015). Many spray programmes employ strobilurins, also known as quinone-outside inhibitor (QoI) fungicides, which act by reducing the respiration capacity of the fungus (Bradley & Pedersen, 2011). Certain *Cercospora sojina* and *Cercospora beticola* strains have shown enhanced resistance against QoI fungicides due to intensive use of these chemicals (Bolton et al., 2013; Zhang et al., 2012). *C. zeae-maydis*, another causal agent of GLS on maize (Wang et al., 1998), has been shown to use alternative respiration pathways to overcome the inhibitory effect of these fungicides (Bradley & Pedersen, 2011). *C. zeae-maydis* or *C. zeina* have not yet shown field fungicide resistance as spray regimes are closely monitored. However, if spray applications were increased, there is a high chance for the development of resistance (Bradley & Pedersen, 2011). Due to the nature of resistance development of pathogenic fungi against most fungicides, there is an increasing need for novel ways to control fungal diseases.

RNA interference (RNAi) is a subcellular gene silencing process that protects organisms against harmful invasions such as viruses (Mitter et al., 2017), and has been shown to also occur in fungi (Catalanotto et al., 2004). Knowledge of RNAi led to a plant protection method called host-induced gene silencing (HIGS; Nowara et al., 2010). Specific small RNAs (sRNA) are expressed within a transgenic plant host to silence genes of invading pests or pathogens (Andrade et al., 2016). The limitation of using HIGS as a disease control method is that it uses genetic modification, making commercialization of products more difficult and time consuming.

Recently, external application of RNAi, a novel way of treating pests and diseases on plants, has been shown to be a promising control strategy. Certain pests, such as the Colorado potato beetle and the xylem-feeding leafhopper, showed increased mortality rates after external treatment of host plants with double-stranded RNA (dsRNA; San Miguel & Scott, 2016). Furthermore, disease severity was reduced in plant hosts of the fungi *Fusarium graminearum*, *Botrytis cinerea* and *Sclerotinia sclerotiorum* after treatment with external dsRNA or sRNA (Koch et al., 2016; McLoughlin et al., 2018; Qiao et al., 2021; Wang et al., 2016). This disease control strategy has been termed spray-induced gene silencing (SIGS) and has been proposed as a sustainable and environmentally friendly disease control strategy (Wang & Jin, 2017).

In this study, we assessed if *C. zeina* fungal cells were able to take up externally applied dsRNA. We tested if the RNA could be used to knockdown GFP fluorescence in a transgenic GFP-expressing *C. zeina* strain. Three *C. zeina*-specific genes were targeted by a dsRNA construct for disruption of gene expression. The metabolic activity of *C. zeina* cells targeted by dsRNA was assessed after in vitro treatment with dsRNA. The timing of germination of *C. zeina* conidia on maize leaves was assessed with microscopy, and dsRNA was assessed for control of GLS disease after inoculation with *C. zeina*.

2 | MATERIALS AND METHODS

2.1 | Fungal and plant materials

Wild-type *C. zeina* isolate CMW25467 (Meisel et al., 2009) and a *C. zeina* transformant expressing GFP (Swart et al., 2017) were cultured on solid V8 medium (Meisel et al., 2009). The GFP-transgenic *C. zeina* was created previously by *Agrobacterium* transformation of wild-type *C. zeina* with a *GFP* expression cassette and the *C. zeae-maydis ctb7* expression cassette (Swart et al., 2017). The V8 medium was supplemented with 50 μg/mL of the cefotaxime analogue Claforan (Sanofi). Cultures were incubated in the dark at room temperature (25°C) and subcultured weekly.

2.2 | RNAi machinery in *C. zeina*

The core genes involved in RNAi processes of *C. zeina* were identified using a phylogenomic approach. The strategy employed was to find the orthologues of the *Neurospora crassa* RNAi machinery proteins, namely Dicer-like 1 (DCL-1; GenBank ID: XP961898), Dicer-like 2 (DCL-2; GenBank ID: KHE87669), Quelling-defective 2 (QDE-2, a type of Argonaute; GenBank ID: XP011394904) and Quelling-defective 1 (QDE-1, an RNA-dependent RNA polymerase; GenBank ID: PKS00983). Orthofinder v. 2.3.1 (Emms & Kelly, 2019) with an inflation factor of 2.5 was used to cluster the proteins from the *N. crassa* genome, the *C. zeina* genome (Wingfield et al., 2022), 99 *Dothideomycetes* fungal genomes (Haridas et al., 2020) and outgroup fungi including two *Eurotiomycetes*, two *Leotiomycetes*, one *Sordariomycete* and one *Pezizomycete* genome. Identified orthologous proteins were functionally annotated by GO (Blast2GO) and PFAM (InterProscan) analyses. Orthologous proteins identified by Orthofinder for each RNAi machinery protein were further curated by removing those that were significantly smaller in size compared to that of the *N. crassa* reference proteins. The *N. crassa* DCL-1 and DCL-2 protein genes are 1584 and 1396 amino acids (AA) in size, respectively, thus any orthologous proteins smaller than 1200

and 1100 AA, respectively, were removed. The QDE-2 proteins are within the range of 1070–1090 AA and any proteins smaller than 900 AA were not included. Additionally, GO and PFAM domains were compared to the reference proteins and those that did not have the same domains as the reference were also removed. The curated protein dataset for each RNAi machinery protein was aligned using Muscle v. 3.8.31. Phylogenetic analyses were conducted using RAxML v. 8.2.1 using 20 maximum-likelihood searches and auto bootstrapping until convergence. Subsequently, a subset of orthologues for each RNAi machinery protein with representatives from each *Dothideomycetes* class including *C. zeina* were used to construct a second set of phylogenetic trees. This was done for ease of visualization of each tree on a single page. The orthologous protein sequences from the selected subset of species were aligned using ClustalW. Phylogenetic analyses were carried out using the maximum-likelihood method with 1000 bootstraps in MEGA X (Kumar et al., 2018). *F. graminearum* and *N. crassa* (class *Sordariomycetes*) proteins were used as out-groups. *C. zeina* gene expression data for the RNAi machinery genes were obtained from GEO RNA-seq datasets GSE90705 (in vitro) and GSE94442 (in planta) (Swart et al., 2017).

2.3 | Synthesis of dsRNA constructs

All primers used in this study are listed in Table S1. A dsRNA construct targeting the *GFP* gene was created through PCR amplification of a 325 bp section of the *GFP* gene, with primers containing T7 overhangs to produce a 365 bp *GFP* DNA template. The *GFP* template was then transcribed into either (a) fluorescent dsRNA (named F-dsRNA-GFP) using the fluorescein RNA labelling mix that incorporates fluorescein-12-uridine triphosphate (fluorescein-12-UTP; Roche Applied Science) or (b) into nonfluorescently labelled GFP-dsRNA using the HiScribe T7 in vitro transcription kit (New England Biolabs). All dsRNA products were purified after transcription using the NucleoSpin RNA kit (Macharey-Nagel).

An RNA construct that targeted three *C. zeina* genes was generated and was called Cz3-dsRNA. The three genes code for the following proteins: chitin synthase D (CHSD; GenBank accession PKS02949.1), phosphatidyl serine decarboxylase proenzyme 3 (PSD3; PKS00696.1) and effector protein 2 (ECP2; PKR94769.1). *C. zeina* gene annotations were obtained from the genome sequence (GenBank accession MVDW00000000) (Wingfield et al., 2017).

C. zeina genomic DNA (gDNA) extraction was carried out with the CTAB extraction method (Meisel et al., 2009) and the gDNA was used as template for subsequent PCRs. The dsRNA construct was created by PCR amplifying a region of genes *CHSD* (798 bp), *PSD3* (404 bp) and *ECP2* (201 bp) separately from the gDNA, and then amplifying them together using an overlapping PCR (Figures S1–S3) (Heckman & Pease, 2007). The final PCR product containing fragments from each of the three genes was then cloned into the pJET1.2/blunt vector using the CloneJet kit (Thermo-Fisher Scientific) to produce plasmid pJET-Cz3. The 1.4 kb insert in pJET-Cz3 was sequenced to assess the integrity of the cloned product. The 1.4 kb Cz3 PCR product was then amplified from pJET-Cz3 using primers containing T7 overhangs (T7-CSD F and T7-PSD R; Table S1), and the product was gel purified using the Zymoclean Gel DNA recovery kit (Zymo Research). The Cz3-dsRNA was obtained from the gel-purified 1.4 kb Cz3 PCR product using the HiScribe T7 in vitro transcription kit (New England Biolabs) following the manufacturer's instructions. The integrity of Cz3-dsRNA was evaluated by cDNA synthesis with the Maxima H Minus First Strand cDNA Synthesis kit (ThermoFisher Scientific), followed by DNA Sanger sequencing using gene-specific primers. The sequenced products were analysed and aligned using CLC Main Workbench v. 8.0.1 software (QIAGEN).

2.4 | In vitro fungal RNA treatments and analysis

The *C. zeina* protoplast extraction protocol was adapted from the *Huntiella* species protocol (Wilson et al., 2020). Briefly, *C. zeina* conidia were harvested from cultures growing on solid V8 medium for a week by washing with double-distilled water. Conidia were incubated in yeast extract-peptone-dextrose (YEPD) medium for 30 h at room temperature, with gentle shaking. The mycelia were collected by filtering the liquid culture through a single layer of sterilized Miracloth (Sigma-Aldrich) and resuspended in 1.2 M KCl. Protoplasts were generated by incubating the mycelia in lysing buffer (500 mg *Trichoderma harzianum* lysing enzyme [Sigma-Aldrich], 25 mL 1.2 M KCl) for 14 h with gentle shaking. The protoplasts were harvested through centrifugation at 1700 *g* for 5 min and resuspended in a STC solution (20% wt/vol sucrose, 50 mM Tris pH 8, 50 mM $CaCl_2$).

For microscopy, the fluorescent dsRNA (F-dsRNA-GFP) was incubated with conidia, mycelia or protoplasts of *C. zeina*. The F-dsRNA-GFP at a final concentration of 1.7 μM was added to 10 μL of 10^6 protoplasts/mL suspended in STC buffer. In addition, F-dsRNA-GFP at a final concentration of 4.2 μM was incubated with 8 μL of 10^6 conidia/mL *C. zeina* conidia or 8 μL of *C. zeina* mycelia suspended in YEPD medium for 14 h with gentle shaking. Fluorescein-12-UTP (Sigma-Aldrich) and double-distilled water were used as negative controls. RNA uptake was observed using an LSM 880 confocal microscope (Zeiss) at the Laboratory for Microscopy and Microanalysis of the University of Pretoria. Excitation and emission wavelengths of 495 and 525 nm, respectively, were used for visualization of fluorescence by GFP and fluorescein-12-UTP. Images were captured and analysed using ZEN 2 software (blue edition; Zeiss).

For dsRNA treatment, *C. zeina* conidia at a concentration of 10^5 conidia/mL (2 mL) were spread with a glass hockey stick onto cellophane disks (Bio-Rad) placed on 1.8% (wt/vol) cornmeal agar (Merck) in plastic Petri dishes (9 cm diameter). The fungal cultures were incubated at room temperature for 7 days. GFP-dsRNA, Cz3-dsRNA (800 ng per plate) or the water control were applied directly onto the fungal cultures, and the plates were incubated for 48 h before RNA extraction as described in Section 2.5. There were three biological replicates per treatment. GFP-transgenic *C. zeina* was used for the

GFP knockdown experiments, whereas wild-type *C. zeina* was used for the Cz3-dsRNA experiments.

For GFP fluorescence activity in *C. zeina* cultures, nonfluorescent dsRNA targeting GFP (GFP-dsRNA) was added at different concentrations (0.3, 0.9 and 2 μM) to 10^5 *C. zeina* conidia/mL in liquid YEPD culture. Total proteins were then extracted from the fungal cultures at 24, 48 and 72 h after addition of dsRNA by homogenizing the fungal mycelia with a mortar and pestle in liquid nitrogen. Powdered samples were transferred into 2 mL Eppendorf tubes with ice-cold 50 mM Tris-HCl (pH 8) and 1 mM PMSF. Samples were centrifuged at 4°C, at 10,600*g* for 10 min. The supernatants containing the protein extracts were collected and analysed further. The concentration of each protein sample was determined using the Bradford protein assay (Bio-Rad). GFP fluorescence was measured at a final protein concentration of 5 μM on a SpectraMax Paradigm (Molecular Devices) at 488 nm excitation wavelength and 535 nm emission wavelength.

2.5 | Fungal RNA extraction, cDNA synthesis and reverse transcription-quantitative PCR

To assess the effect of dsRNA treatments on the expression of target genes (*GFP*, *CHSD*, *PSD3* and *ECP2*), RNA was extracted from the *C. zeina* fungal cultures using the QIAzol lysis reagent (QIAGEN), as per the manufacturer's specifications. The dsRNA-treated fungal cultures had been growing on cellophane disks on cornmeal agar. DNase treatment and RNA purification were carried out using TURBO DNase (ThermoFisher Scientific) and the NucleoSpin RNA kit (Macherey-Nagel). The Maxima H Minus First Strand cDNA synthesis kit (ThermoFisher Scientific) was used to synthesize cDNA, following the manufacturer's specifications, using 1 μg of total RNA and random hexamer primers. Reverse transcription (RT)-PCR was carried out using gene-specific primers and PCR products were Sanger sequenced. The sequenced products were analysed using CLC Main Workbench v. 8.0.1 software (QIAGEN).

The expression of the specific target genes was analysed after the dsRNA treatments using RT-quantitative (q)PCR. The RT-qPCR assay was performed on three biological replicates per treatment using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) following the manufacturer's instructions. Quantification of gene expression was carried out using the CFX Connect Real-Time System (Bio-Rad). Reactions were set up in 10 μL volumes, which consisted of 5 μL of SsoAdvanced Universal SYBR Green Supermix, 0.5 μM of each of the primers, 1 μL of cDNA template and sterile distilled water. The cycling conditions used were 95°C for 10 min; followed by 45 cycles at 95°C for 10 s, 60°C for 15 s and 72°C for 10 s. Fluorescence was measured at the end of each elongation step. Endpoint melt curve analysis was performed to determine that single gene products were amplified. Standard curves of a dilution series of pooled *C. zeina* cDNA was used to evaluate the PCR efficiency and calculate relative quantity (RQ) expression levels for each gene, as described by Hellemans et al. (2007). RQ values for each target *C. zeina* gene were normalized against the stably expressed reference genes *GAPDH* and *40S rRNA* (Swart et al., 2017) to calculate normalized relative quantity (NRQ) values using qBASE^{PLUS} v. 1.0 (Hellemans et al., 2007). Statistical analyses were performed using GraphPad Prism v. 5.04 (GraphPad Software, Inc.).

2.6 | MTT (tetrazolium dye) assay

An MTT assay was used to assess fungal viability after Cz3-dsRNA treatment with three biological replicates per treatment. *C. zeina* conidia (100 μL of 3×10^4 conidia/mL) were added to wells of a clear-bottom 96-well plate. Cz3-dsRNA or GFP-dsRNA at final concentrations of 20 ng/μL was added to the conidia in each well and incubated for 48 h at room temperature. Two foliar fungicides, Nativo (active ingredient strobilurin/demethylation inhibitor; Bayer Crop Sciences) and Artea (active ingredient demethylation inhibitors; Syngenta) at 20 ng/μL were used as positive controls. GFP-dsRNA was applied as a nontarget control and water as the negative control. The MTT cell viability and proliferation assay kit from ScienCell Research Laboratories (Carlsbad) was used. At 48 h after dsRNA inoculation, the MTT reagent was added to each well at a 1/10th volume of the culture volume and incubated for 4 h at 37°C. The supplied solubilization buffer was then added and incubation continued for another hour before the absorbance was measured at 550 nm.

2.7 | Growth chamber inoculations of maize and dsRNA treatments

Inoculations of the susceptible inbred B73 maize line were carried out with modifications of the method described by Meisel et al. (2009). *C. zeina* CMW25467 was cultured on V8 agar plates and conidia were obtained as described in Section 2.4. Maize (*Zea mays*) seeds from inbred line B73 were planted in a mixture of seedling mix potting soil (Culterra) and BlueChem filter sand (Fluidra Waterlinx) in a ratio of 2:1 (vol/vol). Plants were grown at 22 ± 6°C in a plant growth chamber (Conviron) with a day/night photoperiod of 13/11 h and 60% relative humidity (RH). Plants were irrigated daily with 600 mL of water and fertilized every second day with 2 g/L of Solu-Cal Plus (Solu-Cal) and Hygroponic (Hygrotech) fertilizers in a 1:1 (wt/wt) ratio. The dsRNAs were synthesized as described earlier in Section 2.3.

The *C. zeina* conidia were suspended to a final concentration of 5.8×10^5 conidia/mL in a solution of 0.02% Tween 20 (Sigma-Aldrich) in double-distilled water. Maize plants at V7 stage were inoculated with *C. zeina* conidia suspension. A single leaf was chosen at similar height from each maize plant and a 10-cm section along the length of the leaf at 10 cm from the base was marked. An artist's round paintbrush was used to inoculate aliquots of 330 μL of the *C. zeina* conidia suspension on both the abaxial and adaxial sides of the demarcated leaf area. The maize plants were left in darkness

for 3 h prior to and after inoculation. The RH of the chamber was maintained at 80%–85% for a week after inoculation.

The dsRNA treatments were applied to the inoculated leaves either at the time of fungal inoculation (0 hpi) or at 16 hpi. The dsRNAs were diluted with RNase-free double-distilled water and applied at a concentration of 33 ng/μL for Cz3-dsRNA and 15 ng/μL for the GFP-dsRNA. Aliquots of 330 μL dsRNA were applied on both the abaxial and adaxial sides of the demarcated leaf area that was inoculated with *C. zeina*. There were four to six biological replicate maize plants per treatment.

The GLS disease progression was evaluated from the start of lesion development and recorded every 3 days using a GLS disease severity scale of S1–S8, where S1 denotes no symptoms and S8 denotes coalesced necrotic lesions (Table S2). The growth room inoculations of maize and dsRNA treatments were carried out in two independent experiments.

3 | RESULTS

3.1 | RNAi machinery in *Cercospora zeina*

The *C. zeina* CMW25467 genome encoded orthologues of DCL-1 and DCL-2 from *N. crassa* (Figures S4 and S5). The *C. zeina* DCL-1 protein (PKR99831) was 1544 AA in size with 68.8% similarity to the 1584 AA *N. crassa* DCL-1 protein (XP_961898). The *C. zeina* DCL-2 protein (PKR98439) was 1417 AA in size with 59.1% similarity to the 1539 AA *N. crassa* DCL-2 protein (KHE87669). Both *C. zeina* DCL proteins contained the expected functional domains (Catalanotto et al., 2004), namely the N-terminal DEAD-like helicase, the C-terminal helicase, the Dicer dimerization domain and two RNase III domains. Phylogenetic analysis showed that the *C. zeina* DCL-1 and DCL-2 proteins clustered with orthologues from *Cercospora* spp. and related fungi in the order *Mycosphaerellales*, class *Dothideomycetes* (Figures S4 and S5). The highest identities of 93% and 83% were with the *C. zeae-maydis* DCL-1 and DCL-2 proteins, respectively (Figures S4 and S5).

Phylogenetic analysis showed that two *C. zeina* proteins clustered in the same orthogroup as the *N. crassa* Argonaute protein QDE-2 (XP_011394904; 1074 AA) (Figure S6). *C. zeina* AGO-1 and AGO-2 both contained the characteristic PIWI, PAZ, linker and RNase H domains typical of Argonaute proteins. The *C. zeina* AGO-1 protein (PKR99617) of 1016 AA is the most likely functional orthologue of *N. crassa* QDE-2 with 58% sequence similarity, whereas the 1073 AA *C. zeina* AGO-2 protein (PKS00983) had 43% similarity and clustered further from the *N. crassa* QDE-2 on the phylogenetic tree (Figure S6). *C. zeina* AGO-1 grouped with orthologues from other *Cercospora* spp. and other fungi in the order *Mycosphaerellales* (Figure S6), with the highest identity of 87% with the *C. zeae-maydis* orthologue (KAF2215264). *C. zeina* AGO-2 grouped on the phylogenetic tree with proteins from other *Dothideomycete* fungi, including orthologues in *Cercospora* spp. but not *C. zeae-maydis* (Figure S6). A third Argonaute protein of *C. zeina*, AGO-3 (PKS02248; 981 AA), clustered in the orthogroup and shared 64% similarity with the meiotic silencing suppressor QDE-2-like protein of *N. crassa* (XP_958586; 989 AA) (Figure S7). This protein was also conserved in *Cercospora* spp. and other *Dothideomycetes* (Figure S7). The *C. zeina* RNA-dependent RNA polymerase (RdRp; PKS04443; 1379 AA) was the orthologue of the *N. crassa* QDE-1, the RdRp involved in RNAi (EAA29811; 1402 AA) (Figure S8).

RNA-seq data showed that all *C. zeina* RNAi machinery genes described above were expressed in seven in vitro conditions as well as in planta during GLS disease of a susceptible maize line (Table S3). *C. zeina DCL-2* was expressed higher than *DCL-1* in vitro, whereas both were expressed at approximately the 10th percentile of all the fungal genes in planta (Figure S5). The *C. zeina AGO-1* was expressed above the 50th percentile, and more than 5-fold higher than *C. zeina AGO-2* and *AGO-3* in vitro and in planta (Table S3). The *C. zeina RdRp* was expressed at similar levels to *C. zeina DCL-1* in vitro and in planta (Table S3).

3.2 | dsRNA uptake by *C. zeina* and GFP expression knockdown after dsRNA treatment

To assess the capability of *C. zeina* to take up externally applied dsRNA, three different *C. zeina* fungal cell structures, namely protoplasts, mycelia and conidia, were treated for 14 h with F-GFP-dsRNA (a 325 bp fragment of *GFP* labelled with fluorescein-12-UTP). Confocal imaging was used to assess the RNA uptake by wild-type *C. zeina* cells. Micrococcal nuclease (MNase) was applied to remove any F-GFP-dsRNA on the outside surface of the fungal cells. *C. zeina* protoplasts showed significant fluorescent dsRNA uptake, because fluorescence was visible around and inside the cells without MNase treatment, and this remained inside the cells after MNase treatment (Figure 1a). Uptake of F-GFP-dsRNA into *C. zeina* conidia and mycelia was also evident after MNase treatment (Figure S9). The fluorescence inside the *C. zeina* cell types could not be explained by uptake of fluorescein-12-UTP after MNase treatment, because the negative control treatments with free fluorescein-12-UTP did not show fluorescence inside the *C. zeina* cells (Figures S10 and S11).

Transgenic *C. zeina* expressing GFP (Swart et al., 2017) was treated with unlabelled dsRNA that targets the same 325 bp region of *GFP* as F-GFP-dsRNA (Figure S12A). Confocal microscopy was used to assess GFP fluorescence of the GFP-transgenic *C. zeina* with and without GFP-dsRNA treatment (Figure 1b). When comparing fluorescence intensities at either 10× or 100× magnification (Figure 1b), there was less intense fluorescence after treatment with GFP-dsRNA, suggesting that GFP knockdown took place.

Quantitative assessment of the knockdown of *GFP* mRNA expression was carried out by applying the GFP-dsRNA to GFP-transgenic *C. zeina* mycelia. RT-qPCR analysis at 48 h after treatment showed that *GFP* expression was reduced by 57% compared to the water control ($p < 0.05$, *t* test; Figure 2a). GFP knockdown at

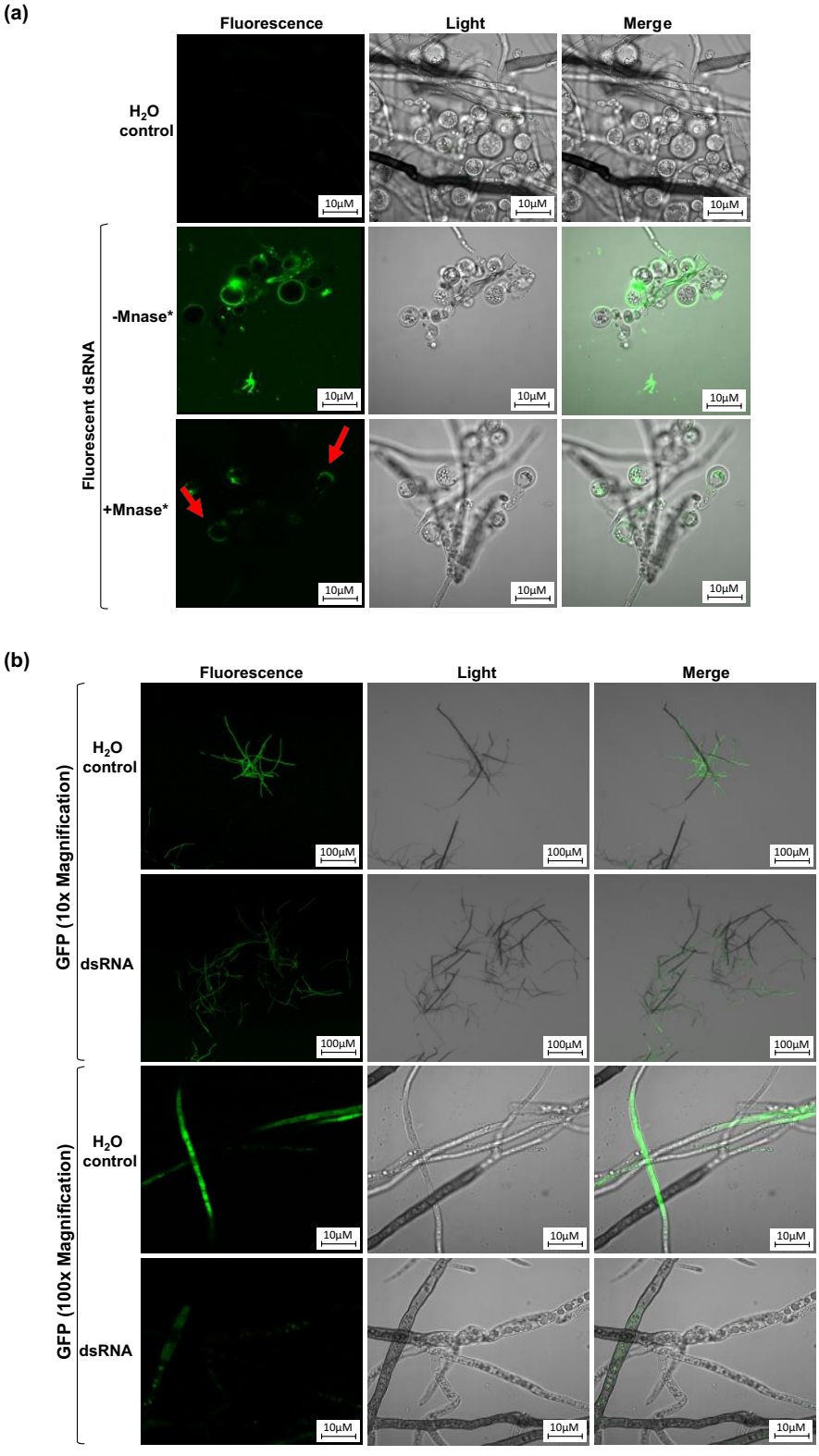

FIGURE 1 Uptake of fluorescently labelled F-dsRNA-GFP by wild-type *Cercospora zeina*, and reduction of *GFP* expression by unlabelled GFP-dsRNA in GFP-transgenic *C. zeina*. (a) *C. zeina* protoplasts took up a fluorescein-12-UTP labelled dsRNA fragment of *GFP* (F-dsRNA-GFP). Uptake of dsRNA into the cytoplasm of *C. zeina* protoplasts was resistant to micrococcal nuclease (MNase) treatment (red arrows). (b) GFP-transgenic *C. zeina* mycelia showed reduced fluorescence when treated with GFP-dsRNA. Water was used as the negative control. Confocal fluorescence microscopy was carried out at excitation/emission wavelengths of 495/525 nm and viewed at 10× (b) and 100× (a and b) magnification. [Colour figure can be viewed at wileyonlinelibrary.com]

the protein level was also evaluated by treatment of GFP-transgenic *C. zeina* with three different concentrations of GFP-dsRNA, and GFP fluorescence was measured at three time points. There was a significant decrease in GFP fluorescence of the GFP-transgenic *C. zeina* treated with 0.9 and 2 μM GFP-dsRNA at 24, 48 and 72 h after treatment compared to the water control ($p < 0.05$, Tukey multiple comparison tests; Figure 2b). The highest knockdown of 61% was seen for the 0.9 μM treatment at 48 h (pTukey <0.05; Figure 2b). The higher concentration of the dsRNA (2 μM) appeared to be less effective than 0.9 μM at the later time points (48 and 72 h). The 0.3 μM treatment decreased GFP fluorescence but the difference was not significant.

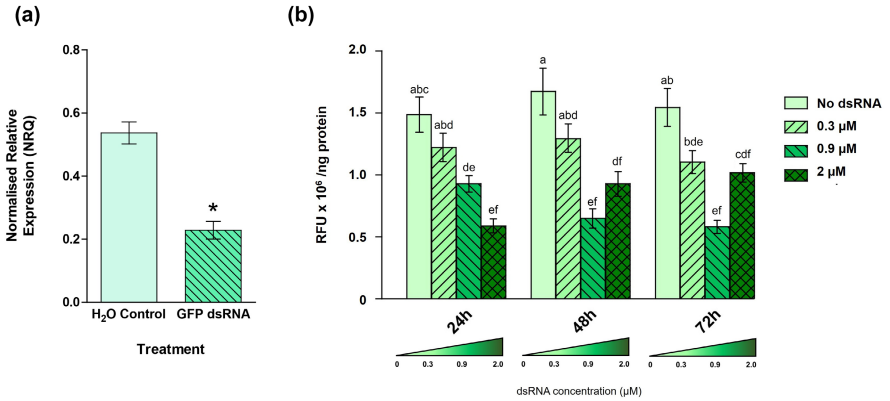

FIGURE 2 Knockdown of *GFP* mRNA and GFP protein levels by treatment of GFP-transgenic *Cercospora zeina* with GFP-dsRNA. (a) Reverse transcription-quantitative PCR of *GFP* expression in GFP-transgenic *C. zeina* at 48 h after treatment with GFP-dsRNA or the water control. Expression levels of *GFP* are reported as normalized relative quantities (NRQ) after normalization against the reference genes, *GAPDH* and *40S rRNA*. Error bars represent the standard error of the mean (*SEM*) (*t* test, $^*p < 0.05$ when compared to the control, $n = 4$). (b) GFP activity in relative fluorescent units of GFP-transgenic *C. zeina* at 24, 48 and 72 h after treatment with 0, 0.3, 0.9 and 2 µM of GFP-dsRNA. Statistical analysis of the data was done using the two-way analysis of variance, which showed a dsRNA concentration effect ($p < 0.0001$), followed by Tukey's multiple comparison tests. Means with the same letters are not significantly different ($p_{Tukey} < 0.05$, $n = 3$). [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | *C. zeina* gene-specific knockdown and metabolic activity after treatment with dsRNA

Three *C. zeina* gene targets were chosen, *CHSD*, *PSD3* and *ECP2* (Table S4). The genes fulfilled the following criteria: (a) a function in either pathogenicity or cell viability; (b) conserved in *Cercospora* species and present in other pathogenic species; (c) used previously in gene knockdown studies in other species; (d) without matches in maize, checked with tBLASTn against the B73 maize RefGen_v4 genome; (e) expressed in planta with a $\log_2$ counts per million (CPM) >2 (GEO dataset: GSE94442); (f) expressed in vitro on V8 sporulation medium and cornmeal agar at greater than the 50th percentile of all genes (GEO dataset: GSE90705); and (g) genes present in single copy in the *C. zeina* genome.

The plasmid pJET-Cz3 containing sections of the *C. zeina CHSD* (798 bp), *PSD3* (404 bp) and *ECP2* (201 bp) genes was constructed (Figures S1 and S2), and used to generate the 1443 bp template with T7 promoters on each end for in vitro synthesis of Cz3-dsRNA (Figure S3). The product size of Cz3-dsRNA on a 0.8% agarose gel migrated to the equivalent double-stranded DNA (dsDNA) ladder size of 1000 bp and not the expected 1403 bp (Figure S12B, lane 2). RT-PCR and sequencing analysis of Cz3-dsRNA showed that the complete sequences of *CHSD*, *ECP2* and *PSD3* were present. The migration of dsRNA of this size range appears to be faster than the double-stranded DNA ladder standards because the 1800 bp dsRNA control synthesized with the RNA synthesis kit migrated to 1200 bp (Figure S12B, lane 3). The formation of RNA secondary structures is a possible explanation for the differences in gel electrophoresis between dsRNA and the dsDNA ladder.

C. zeina growing on cornmeal agar was inoculated with either water or the Cz3-dsRNA and after 48 h the total RNA was extracted. RT-qPCR analysis was conducted on each specific gene, using *GAPDH* and *40S rRNA* as reference genes. The *C. zeina CHSD* gene expression showed a significant decrease of 47% after Cz3-dsRNA treatment compared to the water control ($p < 0.05$, *t* test; Figure 3a). Cz3-dsRNA treatment resulted in a 20% reduction of *C. zeina PSD3* gene expression; however, this reduction was not significantly different from the water control (Figure 3a). The *C. zeina ECP2* was expressed at a low level under these conditions and there was no difference between treatments (Figure 3a).

An MTT assay was used to assess the effect of dsRNA treatment on the metabolic activity of *C. zeina* in vitro. *C. zeina* conidia were treated with Cz3-dsRNA, GFP-dsRNA or water for 48 h and the metabolic activity of the cells was analysed by adding the colourimetric substance MTT. The Cz3-dsRNA treatment resulted in a significant 34% decrease in the fungal metabolic activity compared to the water-treated samples ($p < 0.001$, Dunnett's multiple comparison test; Figure 3b). The GFP-dsRNA treatment did not show a significant difference from the water treatment (Figure 3b). Two fungicides (Nativo and Artea) were used as positive controls. *C. zeina* treated with each of the two fungicides showed significant decreases in cell metabolic activity of up to 51% compared to the water treatment ($p < 0.001$, Dunnett's test; Figure 3b).

3.4 | Germination of *C. zeina* conidia on maize leaves

The F-dsRNA-GFP uptake experiments indicated that *C. zeina* conidia took up dsRNA less efficiently than mycelia and protoplasts (Figure 1a; Figure S9). We hypothesized that dsRNA control of *C. zeina* may be more effective after germination on the maize leaf surface. Considering that *C. zeina* has a long latent period before symptoms appear (Meisel et al., 2009), it was not known if the fungus germinates and penetrates soon after landing on a leaf surface or it remains on the surface for some time. Leaves of the susceptible maize genotype B73 were inoculated with wild-type *C. zeina*. Scanning electron microscopy (Figure S13) revealed that 70.6% of conidia germinated by 16 hpi,

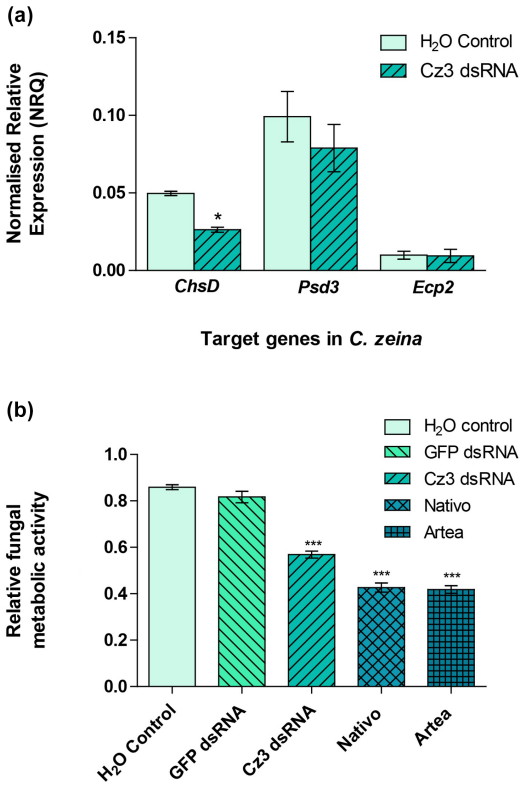

FIGURE 3 Treatment of *Cercospora zeina* with Cz3-dsRNA reduces target gene expression and fungal cell viability. (a) Reverse transcription-quantitative PCR of target genes in wild-type *C. zeina* at 48 h after treatment with Cz3-dsRNA shows significant reduction of *CHSD* but not *ECP2* or *PSD3* expression. Expression levels of each gene are reported as normalized relative quantities (NRQ) after normalization against the reference genes, *GAPDH* and *40S rRNA*. Error bars represent the standard error of the mean (SEM) (*t* test, $^{*}p < 0.05$ when compared to the water control, $n = 4$). (b) MTT viability assays of wild-type *C. zeina* at 48 h after dsRNA and fungicide treatments. MTT absorbance measurements were taken at 570 nm. Statistical analysis of the data were done using the one-way analysis of variance, which showed a treatment effect ($p < 0.0001$); followed by the Dunnett's multiple comparison tests ($^{***}p < 0.001$ when compared to the water control, $n = 3$; error bars represent ± *SEM*). [Colour figure can be viewed at wileyonlinelibrary.com]

increasing to 77.3% at 24 hpi (Table S5). Positive tropism of *C. zeina* germ tubes towards maize stomata was also observed (Figure S13). Light microscopy (Figure S13) of in vitro control samples showed that only 6.5% of conidia were germinated at 0 h in water, and only reaching 33.2% at 16 h. In contrast, 72.4% of *C. zeina* conidia germinated at 16 h on V8 agar, which is known to stimulate germination of this fungus (Meisel et al., 2009), and this was significantly different from the water treatment at 16 h ($p < 0.05$, Mann–Whitney *U* test; Table S6).

3.5 | Reduction of GLS disease in maize leaves by dsRNA targeting *C. zeina* genes

Based on the microscopy results, it was decided to apply the Cz3-dsRNA and the negative control GFP-dsRNA treatments at 0 or 16 h after inoculation of maize leaves with the wild-type *C. zeina*. GLS disease symptoms appeared at 22 days post-inoculation (dpi) on the control *C. zeina*-inoculated maize B73 plants that had not been treated with dsRNA. This is consistent with previous growth room assays of this pathosystem, as *C. zeina* has a long latent period of 2–3 weeks (Meisel et al., 2009). The first symptoms were the expected small chlorotic spots, which developed into mature lesions in the control plants over the course of each trial, and disease was scored at six time points to calculate area under the disease progress curve (AUDPC) for each treatment. GLS disease was not significantly different between leaves treated with water, GFP-dsRNA or Cz3-dsRNA at $t = 0$ hpi (the same time as *C. zeina* inoculation) for both independent trials ($p > 0.05$, Mann–Whitney *U* test; Figure 4a,b). However, GLS disease was significantly reduced when the Cz3-dsRNA was applied at 16 hpi, with 56% and 39% reductions on average in the first and second trials, respectively ($p < 0.05$, Mann–Whitney *U* test; Figure 4a,b). Importantly, the GFP-dsRNA treatments did not reduce GLS disease symptoms when applied at either 0 or 16 hpi in both trials, and disease scores were not significantly different from the water treatments ($p > 0.05$, Mann–Whitney *U* test; Figure 4a,b).

4 | DISCUSSION

The overall aims of this study were to evaluate if *C. zeina* can take up externally applied dsRNA and whether this can lead to GLS disease control on maize leaves. The main findings from this study were that (a) *C. zeina* has the core RNAi machinery genes, and they are expressed in vitro and in planta; (b) *C. zeina* was able to take up externally applied dsRNA; (c) the dsRNA application resulted in knockdown of specific gene expression in *C. zeina* and reduced fungal cell viability; and (d) externally applied dsRNA targeting *C. zeina* genes but not *GFP* was able to reduce GLS disease symptoms in planta.

An important contribution of this work was to demonstrate that *C. zeina*, and other related fungi in the order *Mycosphaerellales*, class *Dothideomycete* have the core genes for RNAi, namely *DCL-1*, *DCL-2*, *Argonaute* and *RNA-dependent RNA polymerase*. Interestingly, *C. zeina* has three copies of *Argonaute*, similar to *Pyrenophora tritici-repentis*, and *Leptosphaeria maculans*, whereas other *Dothideomycetes* such as *Botryosphaeria dothidea* and *Zymoseptoria tritici* have four copies each (Hu et al., 2019; Kettles et al., 2019). In *Z. tritici* only two copies of the Argonaute genes, *ZtAGO1* and *ZtAGO2*, appeared to be functional while the other two are missing essential N-terminal domains (Kettles et al., 2019). Therefore, future work will include determining which copies of *DCL-1* and *AGO* are involved in dsRNA-mediated gene silencing in *C. zeina*. Having multiple copies of these genes might favour the fungal RNAi system. Some fungi such as *Z. tritici*, which has only one *DCL* gene, are compromised in this activity (Kettles et al., 2019).

The uptake of fluorescently labelled dsRNA by *C. zeina* protoplasts was observed as fluorescence in the cytoplasmic material. Fluorescently labelled dsRNA was pressed by turgor pressure of the

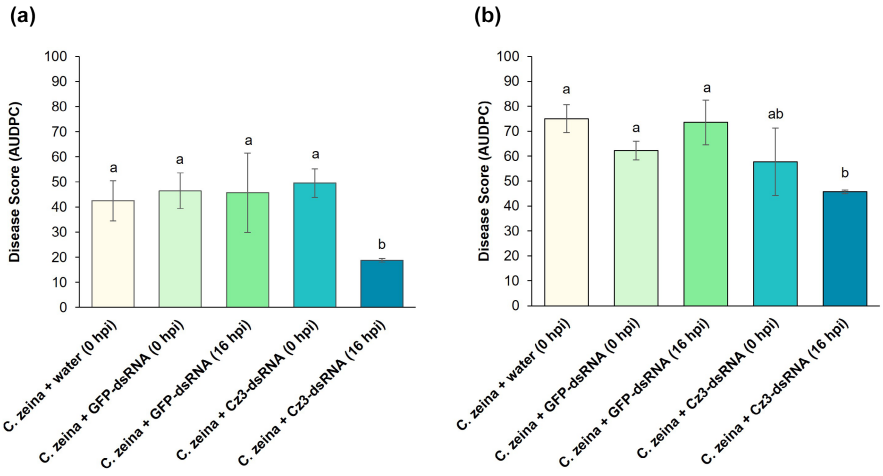

FIGURE 4 Grey leaf spot (GLS) disease is reduced by treatment with Cz3-dsRNA on *Cercospora zeina*-inoculated maize leaves. GLS disease severity scores for each treatment are shown for two independent trials (a and b). The bar graphs show the GLS disease severities quantified as area under the disease progress curve (AUDPC) for each treatment. The treatments included *C. zeina* water-treated control (*C. zeina* + water, 0 h post-inoculation [hpi]); the GFP dsRNA specificity negative control (*C. zeina* + GFP-dsRNA, 0 hpi); the dsRNA targeting three genes of *C. zeina* (*C. zeina* + Cz3-dsRNA, 0 hpi); and applications of the dsRNA at 16 hpi, namely *C. zeina* + GFP dsRNA (16 hpi); and *C. zeina* + Cz3-dsRNA (16 hpi). In both trials, inoculated maize leaves treated with Cz3-dsRNA at 16 hpi showed significant GLS disease reduction compared to the water control and GFP-dsRNA. 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 ($\alpha = 0.05$, $n = 4–6$, error bars represent *SD*). [Colour figure can be viewed at wileyonlinelibrary.com]

vacuoles to the inside of the periphery of the protoplasts and was resistant to MNase digestion. These results were similar to what was observed in *B. cinerea* and *S. sclerotiorum* (Qiao et al., 2021; Wang et al., 2016), and indicated that the dsRNA did not move into the vacuoles.

The choice of pathogen target genes is important for dsRNA-mediated gene silencing and SIGS, and therefore we developed a gene selection process like the 'target identification pipeline' (TIP) for *S. sclerotiorum* employed by McLoughlin et al. (2018). In our study there was effective gene silencing of *GFP* in the GFP-transgenic *C. zeina*. We also obtained silencing in wild-type *C. zeina* of one of the three genes in the *C. zeina* triple gene construct, namely *CHSD*. Other studies have also reported variation in effective silencing of different genes; for example, there was significantly greater silencing of *FgCYP51A* than two other *CYP51* genes in the *F. graminearum*–*Arabidopsis* pathosystem (Höfle et al., 2020). The lack of significant downregulation of *C. zeina PSD3* and *ECP2* could be due to their expression levels. *PSD3* was expressed at a higher level than *CHSD* in the water control treatment, and thus there may have been insufficient dsRNA molecules to silence all the *PSD3* transcripts. In contrast, the *ECP2* expression levels may have been too low and variable under these conditions to detect a significant silencing effect. McLoughlin et al. (2018) used a detached leaf assay of brassica plants to screen dsRNA targeting 59 *S. sclerotiorum* genes, and dsRNA of only 20 genes showed a significant disease control due to silencing, indicating that target selection is crucial.

The length of the dsRNA has also been shown to be important for SIGS. In the *F. graminearum*–barley interaction, silencing was less effective when full-length *CYP51* gene dsRNA (1.5 kb) was applied compared to shorter dsRNA of less than 800 bp (Höfle et al., 2020). The most effective silencing was observed with 200–300 bp dsRNA of *F. graminearum CYP51* genes (Höfle et al., 2020), and in previous work a triple *CYP51* gene construct of 791 bp proved effective (Koch et al., 2016). The authors proposed that the longer dsRNA may be taken up less efficiently in SIGS, because the same constructs expressed in HIGS (where the dsRNA is synthesized in planta) were not affected by construct length (Höfle et al., 2020). In our study, we observed highly effective silencing of *GFP* by a 325 bp dsRNA in vitro despite high expression of the target gene (Swart et al., 2017). The Cz3-dsRNA construct that we employed for *C. zeina* control was 1.4 kb in size, which may have been taken up less efficiently, as seen in *F. graminearum*. Future work with a shorter construct could result in improved silencing and disease control.

We observed that the dose of dsRNA influenced silencing of *GFP* in the GFP-transgenic *C. zeina*. We obtained the highest gene silencing after 48 hpi when GFP-dsRNA was applied at a concentration of 0.9 μM. A higher concentration of 2 μM GFP-dsRNA initially showed good silencing at 24 hpi, but as time progressed, the silencing capabilities at this high concentration was lost. In other studies, a saturation effect was also observed with using increasing doses of dsRNA (McLoughlin et al., 2018; Mitter et al., 2017). It is thought that at higher dosages the RNAi silencing machinery could be saturated with the excess of RNA molecules, and cleavage would be rate-limiting (McLoughlin et al., 2018).

Chitin is an essential molecule forming part of the carbohydrate skeleton of the fungal cell wall (Lenardon et al., 2010), and fungi have evolved diverse effectors to avoid recognition of chitin by

plant hosts (Rocafort et al., 2020). The importance of chitin biosynthesis for fungal cell viability was highlighted in our study because silencing of the *C. zeina CHSD* with dsRNA reduced the cell viability in vitro. In previous work, HIGS targeting chitin synthase genes was shown to be effective in disease control against *S. sclerotiorum* and *F. graminearum* (Andrade et al., 2016; Cheng et al., 2015).

It is plausible that the *C. zeina* disease control that we observed from the treatment of maize leaves with Cz3-dsRNA was therefore on account of silencing of the fungal chitin synthase gene *CHSD* by this part of the triple gene construct in both growth chamber trials. This effect was specific because GFP-dsRNA treatments did not reduce disease, like the control treatments used in the *S. sclerotiorum–Brassica* experiments (McLoughlin et al., 2018). The level of *C. zeina* disease control that we observed, namely 56% in the first trial and 39% in the second trial, was within the range seen in previous studies with the application of naked dsRNA. Lesion sizes on fruits of tomato, strawberry and grape and lettuce leaves caused by *B. cinerea* were reduced by up to 80% by application of dsRNA targeting *DCL-1* and *DCL-2* of the pathogen (Wang et al., 2016). *F. graminearum* DNA content measured with an in planta qPCR assay was reduced by 42%–78% when barley leaves were treated with the *CYP3*-dsRNA (Koch et al., 2016). Lesion size caused by *S. sclerotiorum* was reduced on *Brassica napus* leaves by 26%–86% with application of dsRNA targeting different genes (McLoughlin et al., 2018). Potato leaf infection area from inoculation with *Phytophthora infestans* was reduced by 44%–73% with dsRNA targeting three different genes (Kalyandurg et al., 2021).

All of these examples and most other SIGS studies that have been carried out to date have used detached leaf assays with necrotrophic pathogens where disease development and the dsRNA effect can be scored within a few days (e.g., Koch et al., 2016; McLoughlin et al., 2018). In contrast, the *C. zeina*–maize pathosystem is more challenging because mature plants close to flowering are required for disease development, and there is a latent period of 2–3 weeks before symptoms are evident (Swart et al., 2017). Furthermore, it appears that dsRNA uptake is more efficient after *C. zeina* conidia have germinated, and thus dsRNA treatment was required 16 hpi for effective disease control. Beckman and Payne (1982) reported that spore germination in *C. zeae-maydis* occurs at 24 hpi, supporting the finding from this study in which efficient dsRNA application was observed after 16 hpi. The less efficient uptake may be related to the size of the construct (1.4 kb), as was shown in *F. graminearum* (Höfle et al., 2020). Therefore, testing shorter constructs and targeting additional *C. zeina* metabolic processes such as ergosterol biosynthesis would be future priorities for research.

This study lays the groundwork for the development of an RNAi-based fungicide against *C. zeina* that causes GLS, a yield-limiting foliar disease of maize. We established that this fungus and related plant-pathogenic *Dothideomycetes* fungi have the core RNAi machinery and that *C. zeina* cells take up externally applied dsRNA, which reduces disease symptoms. Future studies should assess if carrier molecules such as nanoparticles can stabilize the dsRNA and improve uptake by the fungus in field settings (Mitter et al., 2017).

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any financial or commercial relationship that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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