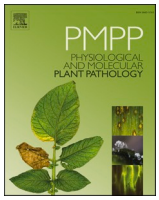
Physiological and Molecular Plant Pathology 143 (2026) 103161

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp



Functional RNAi response in *Colletotrichum graminicola* reveals potential for host-induced gene silencing against maize anthracnose

Raquel Pereira Passos Salgado [a], Gabriel Angelo Saraiva Raimundo [b], Lucas Romão Gava [c], Andréa Almeida Carneiro [b], Fernando Hercos Valicente [b], Dagma Dionísia da Silva [b], Luciano Viana Cota [b], Meire de Cássia Alves [b], André da Silva Xavier [d], Newton Portilho Carneiro [b,*]

[a] Universidade Federal de Lavras, Lavras, 37203-202, Minas Gerais, Brazil
[b] Embrapa Milho e Sorgo, Núcleo de Biologia Aplicada, Sete Lagoas, 35702-098, Minas Gerais, Brazil
[c] Universidade Federal de Viçosa, Campus Viçosa, Viçosa, 6570-075, Minas Gerais, Brazil
[d] Universidade Federal Rural de Pernambuco, Recife, 52171-900, Pernambuco, Brazil

ARTICLE INFO

Keywords:
dsRNA
Phytopathogenic protection
siRNA
Topical application of dsRNA
Transgenic maize

ABSTRACT

Colletotrichum graminicola is a filamentous fungus that causes anthracnose disease in maize, leading to yield losses of up to 35%. RNA interference (RNAi) has been successfully employed to protect crops against phytopathogenic fungi through multiple approaches, demonstrating considerable efficiency in silencing target genes. In this study, it was evaluated RNAi-mediated silencing of *C. graminicola* chitin synthase genes (*CHSV* and *CHSVII*), two important structural components and the phosphopantetheinyl transferase gene (*PPT*) involved in the melanin biosynthesis pathway and directly associated with *C. graminicola* pathogenicity. In this work, it was also tested siRNA and dsRNA and topical applications in culture medium and detached maize leaves. The siRNAs in culture media proved more efficient in reducing transcripts levels compared to long dsRNAs; and in detached maize leaves it was observed the opposite. No significant difference was found in the overall suppressive effect of siRNAs and dsRNAs, as both approaches inhibited fungal biomass accumulation and reduced the severity of anthracnose in maize leaves. It was also tested the effect of expression CHSVII dsRNA in transgenic maize with experimentally infected *C. graminicola*. The 20 maize transgenic events showed a gradient from low to high severity of anthracnose in leaves and stalk compared to the non-transgenic plants indicated that it also can be used as strategy to mitigate this disease. The RNAi strategies applied in this study to inhibit *C. graminicola* growth both *in vitro* and *in vivo*, as well as to protect transgenic maize plants against anthracnose, suggest this could support future technologies aimed at mitigating yield losses and reducing fungicide application used in maize fields.

1. Introduction

The genus *Colletotrichum* is currently classified among the ten most important plant pathogenic fungi worldwide due to its substantial economic impact and high relevance to plant pathology research [1,2]. Species within this genus are responsible for anthracnose, a disease that affects a wide range of plant hosts, including tropical and temperate crops, as well as ornamental, woody, and herbaceous species [3–8]. The *C. graminicola* is capable of causing severe damage to maize plant and exhibit considerable genetic diversity. In a study evaluating monosporic isolates of *C. graminicola* collected from infected maize leaves across seven Brazilian ecogeographic regions, four out of five isolates were shown to be highly virulent against 15 tropical maize genotypes [9].

C. graminicola produces hyaline, sickle-shaped, aseptate conidia, that are formed in highly melanized structures called acervuli, which are surrounded by spiky projections [10]. During the infection, the conidia land on the leaf surface and develops a specialized structure called an appressorium, responsible for penetrating the leaf epidermis. Melanin

* Corresponding author.
E-mail addresses: rppsalgado@gmail.com (R.P.P. Salgado), gabriel88saraiva@gmail.com (G.A.S. Raimundo), lucas.romao@ufv.br (L.R. Gava), andrea.carneiro@embrapa.br (A.A. Carneiro), fernando.valicente@embrapa.br (F.H. Valicente), dagma.silva@embrapa.br (D.D. Silva), luciano.cota@embrapa.br (L.V. Cota), meire.alves@embrapa.br (M.C. Alves), andre.sxavier@ufrpe.br (A.S. Xavier), newton.carneiro@embrapa.br (N.P. Carneiro).

https://doi.org/10.1016/j.pmpp.2026.103161
Received 1 August 2025; Received in revised form 5 February 2026; Accepted 17 February 2026
Available online 21 February 2026
0885-5765/Published by Elsevier Ltd.

plays a crucial role at this stage, by increasing the turgor pressure within the appressorium, enabling the fungus to breach the leaf cuticle. Consequently, melanin is considered a key virulence factor in *C. graminicola* pathogenicity [11]. The life cycle of *C. graminicola* is characterized by the alternation between two lifestyles: biotrophic and necrotrophic. In the biotrophic phase, the fungus establishes a parasitic relationship with the host cells, while in the necrotrophic phase, it induces progressive cell death and the development of extensive lesions, ultimately leading to plant death [12]. The characteristic symptoms of anthracnose include stem rot and necrotic lesions on the leaves [13].

The use of fungicides remains one of the most common and effective strategies for controlling fungal diseases in crops with economic importance. However, their extensive and repetitive use has increased resistant fungal populations, environmental contamination, and impacts on non-target organisms. Sustainable management practices using biological molecules such as dsRNA, have become essential to enhance disease control while reducing environmental risks.

RNA interference (RNAi), also known as post-transcriptional gene silencing, is an intrinsic and conserved regulatory mechanism in eukaryotic organisms [14]. It functions by processing double-stranded RNA (dsRNA) fragments into small interfering RNAs (siRNAs), which guide the degradation of complementary target transcripts [15]. RNAi has become a widely used tool in functional genomics [16] and has shown promising results in research aimed at increasing the resistance of crops plant to pathogens [17,18]. Several approaches have been developed to achieve this purpose. One such approach involves the *in vitro* synthesis of dsRNA fragments and their direct delivery to the target organism using techniques such as Spray-Induced Gene Silencing (SIGS) [19–21]. Additionally, genetic engineering techniques have enabled the development of methods like Host-Induced Gene Silencing (HIGS) and Virus-Induced Gene Silencing (VIGS). These transient transformation methods have successfully reduced the infectivity of various phytopathogens in different crop cultivars [22–26].

RNA interference (RNAi) demonstrated successful control of insects, nematodes, viruses and parasitic plants [19]. Despite the success of RNAi approaches to control some organisms, there are not too many successful cases to manage phytopathogen fungi especially for anthracnose in maize. It is important to consider different methods of topical application as the effect of expression dsRNA in transgenic maize lines.

Genes involved in primary and secondary metabolism that are essential for fungal pathogenicity, such as phosphopantetheinyl transferases (PPTases) and polyketide synthases (PKSs), have been identified as potential targets for controlling plant infections. PKSs are post-translationally activated via a phosphopantetheinyl transferase reaction catalyzed by PPTases. These enzymes also regulate components of primary metabolism in fungi [27]. In *Colletotrichum* spp. and *Magnaporthe oryzae,* PKS-derived melanin is required for generating turgor pressure in appressoria, and gene knockout studies have shown a direct loss of pathogenicity when melanin biosynthesis is disrupted [28,29].

Chitin synthases (*CHS*) represent an important group of enzymes involved in the synthesis of chitin, a polymer (1,4 N-acetylglucosamine). Chitin is a key structural component of the fungal cell wall providing shape and rigidity to fungal cells [30]. CHS constitutes a large family of isoenzymes classified into distinct classes based on amino acid sequences similarity, and are encoded by the genes such as *CHSI*, *CHSII*, *CHSIII*, *CHSV* and *CHSVII* [31]. The chitin biosynthesis pathway is a well-established target for the development of new fungicides aimed at controlling phytopathogenic fungi in the field [32]. Therefore, silencing specific *CHS* genes in *C. graminicola* represents a promising strategy for controlling anthracnose in maize.

Here, it is explored topical applications of three different genes (*CHSV*, *CHSVII*, and *PPT)*, two different molecules and two different conditions (cultural media e leave tissue) against *C. graminicola*. Additionally, we algo determined the impact of overexpression of the gene (*CHSVII)* in transgenic maize to control *C. graminicola.* The findings of this study contribute to the development of RNAi-based strategies for controlling this phytopathogenic fungus in maize.

2. Material and methods

2.1. Fungal isolates, pathogenicity test, mycelial growth and conidiation

Monosporic isolates of *C. graminicola* (21.10 and 10.09) were obtained from Microorganisms Stock Collection at Embrapa Maize and Sorghum, Sete Lagoas, Minas Gerais, Brazil. Molecular confirmation of the *C. graminicola* isolates was done by sequencing (Hitachi 3500 Genetic Analyzer®) and analyzing via BLASTn (Basic Local Alignment Search Tool) of the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *ITS* (internal transcribed spacer), and *β-tubulin* genes using gene-specific primers (Table S1) PCR amplicons [33–35]. The pathogenicity of the isolates was confirmed by inoculating the susceptible maize genotype BRS1010 with a conidial suspension at a concentration of 10^6 conidia/mL, and the symptoms of anthracnose were evaluated 10 days after inoculation. Sporulation of *C. graminicola* isolates was induced on Oat Flour Agar (OFA) medium. All the experiments were done using the monosporic isolates of *C. graminicola* (10.09).

2.2. Identification and in silico characterization of RNA silencing components

The main genes involved in RNAi machinery in *C. graminicola* were identified using a comparative phylogenetic analysis to other phytopathogen fungi. The strategy identified orthologs (or homologs) of closed related species RNAi machinery. Putative homologs of the RNA interference (RNAi)-related proteins Argonaute (AGO), Dicer-like (DCL), and RNA-dependent RNA polymerase (RDR) in Ascomycota fungi were identified using BLASTP searches. Protein sequences from *C. higginsianum* were used as queries to retrieve corresponding sequences from *C. graminicola* genome, as well as from other *Colletotrichum* species. Candidate proteins were identified by evaluation of conserved domains using the CNBI Conserved Domain Database (CDD). Specifically, RDR proteins were required to contain an RNA-dependent Polymerase Domain, DCL proteins had to possess two RNaseIII-domains; and AGO proteins were identified based on the presence of both PAZ and PIWI domains.

The deduced amino acid sequences for the RNA silencing proteins (Table S2) were aligned with CLUSTAL W [36]. The aligned sequences were then imported to Molecular Evolutionary Genetics Analysis (MEGA v.7.0.21) [37]. The best fit model of protein evaluation was performed using MEGA's model (v.7.0.21). The LG + G model was determined using MEGA model selection tool, to be the best adjustment for protein sequences. Maximum Likelihood (ML) phylogenetic trees were subsequently constructed in MEGA (v.7.0.21) using this LG + G model [38].

The resulting trees were visualized using Inkscape (https://inkscape.org/>. Access Jan. 2019) Draw Freely (v. 0.92) with a radial format, and only branches with bootstrap support values above 90% were retained. *C. graminicola* RNA silencing proteins were compared to those previously reported for *C. higginsianum* [39].

2.3. Selection of target genes, primer design for RNAi and synthesis of dsRNA

Three targeted genes were selected for *in vitro* transcription (dsRNA synthesis). These included two genes involved in chitin biosynthesis, Chitin Synthase V (*CHSV*) (XM_008099003.1 - NCBI) and Chitin Synthase VII (*CHSVII*) (XM_008093411.1 - NCBI), and the third is associated with melanin biosynthesis, Phosphopantetheinyl transferase 1 (*PPT1*) (XM_008092455.1 - NCBI). For the isolation of RNA, *C. graminicola* strain 10.09, was grown in Potato Dextrose Agar (PDA) liquid medium and the RNA extract using the Pure Link RNA mini-Kit (Thermo Fisher Scientific - Waltham, Massachusetts, USA) following the manufacturer's

instructions. RNA concentration was determined using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific - Waltham, Massachusetts, USA), and RNA integrity was verified by electrophoresis on a 2% (w/v) agarose gel. First-strand cDNA synthesis was performed from 1 μg of total RNA using the High-Capacity RNA-to-cDNA ™ Kit (Thermo Fisher Scientific - Waltham, Massachusetts, USA), according to the manufacturer's protocol. Conventional PCR was carried out using the previously synthesized cDNA as a template. Gene specific forward and reverse primers (Table S1) were designed with the T7 promoter sequence (5′-TAATAACTCACTATAGGG-3′) incorporated at the 5′end of both primers. dsRNA synthesis was performed using MEGAscript™ T7 Transcription Kit® (Thermo Fisher Scientific - Waltham, Massachusetts, USA) using 1 μg of purified PCR product. Transcription reactions were incubated at 37 °C for 16 h. Following synthesis, the dsRNA was evaluated on a 1% agarose gel, quantified, and diluted to the appropriate concentration for subsequent experiments. For the primer efficiency test (Table S1) was determined using a 1:5 serial dilution factor and a five-point standard curve. Primer efficiencies (EFF%) between 81% and 110% were considered acceptable. Specificity was confirmed by the presence of a single melting curve peak. The reference gene used for normalization was *β-tubulin* [40], and amplicon sizes ranged from 80 to 100 bp. For GFP dsRNA synthesis, PCR product used the plasmid B437 (DNA Cloning Service E. K., Hamburg, Germany) as template. The relative expression was performed on the Applied Biosystems® 7900HT Fast Real-Time PCR System, and the reaction was prepared with SYBR™ Select Master Mix (Thermo Fisher Scientific - Waltham, Massachusetts, USA). Data analysis was performed to calculate the mean Relative Quantification (RQ) values. Small interfering RNAs (siRNAs) were generated *in vitro* by enzymatic cleavage of dsRNA fragments using the ShortCut® RNase III enzyme (New England Biolabs (NEB) - Ipswich, Massachusetts, USA).

2.4. Effect of gene silencing on the development of C. graminicola in vitro

Bioassays were carried out in 24-well cell culture plates, each containing 2 ml of Potato Dextrose Agar (PDA) medium. For the dose-response assay, siRNAs were applied at final concentrations of 0.5, 1.0 and 2.0 μg. For the other *in vitro* assays, a final concentration of 1.0 μg of either dsRNA or siRNA was used. Conidial suspensions were prepared from the monosporic *C. graminicola* isolate 10.09 in Oat Flour Agar media under continuous light for 7-8 days. The conidial suspension (10^4 conidia/mL) was treated with the dsRNA and siRNA at the same final concentrations (0.5, 1.0, and 2.0 μg) in the dose-response assay, and 1 μg for all the other experiments. The plates were maintained at 25°C with a 12-h photo period. After five days of fungal growth, the treatments were evaluated.

The effects of *CHSV*, *CHSVII, PPT* dsRNAs and siRNAs on *C. graminicola* gene silencing and disease severity in maize plants were evaluated at the V3 developmental stage of the susceptible maize genotype BRS1010. The plants were maintained at 25°C with a 12-h photo period. After five days of fungal growth, the treatments were evaluated. Five plants were used for each target gene, GFP dsRNA (dsGFP) and dsGFP-derived siRNA (siGFP) were used as negative controls. RNA was extracted from actively growing fungal mycelia ground in liquid nitrogen, using Pure Link RNA Minikit (Thermo Fisher Scientific - Waltham, Massachusetts, USA), following the manufacturer's protocol.

The evaluation of *C. graminicola* mycelial biomass was performed by incubating a mycelial disk in microtubes containing either dsRNA or siRNA solution (final concentration 1 μg), for 48 h at 25 °C under agitation. Subsequently, the mycelial discs were added to 10 mL of Potato Dextrose Broth (PDB) medium and incubated under agitation for eight days at 25 °C. After incubation, the mycelium was dried at room temperature (25 °C) for two days and weighed on a precision scale.

2.5. Plant RNAi vector construction and cloning

A construct containing two inverted copies (311 bp each) of the *CHSVII* gene from *C. graminicola*, separated by a 513 pb intron sequence and driven by the maize Ubiquitin promoter (modified with the second intron of the potato ST-LS1 gene), was synthesized and cloned in the vector pBS (DNA Cloning Service E. K., Hamburg, Germany). Subsequently, the construction was subcloned into the p7i2x-UbiZm vector using the restriction sites of the *Eco*RI and *Sal*I enzymes to generate p7i2x-UbiZm:dsCHSVII and transformed into *Agrobacterium* EHA105.

2.6. Transformation of maize plants

Maize HiII immature embryos were transformed with the p7i2x-UbiZm:dsCHSVII vector via *A. tumefaciens* strain EHA101 [41,42]. Thirty putative transgenic T0 plants were confirmed in the greenhouse by PCR using primers targeting the *bar* and *CHSVII* genes (Table S1). The T0 plants were crossed with a non-transgenic L3 line plant, and the progeny of twenty showed a segregation of 1:1 ration of the herbicide resistant/nonresistant (Finale® 1.0 mg/L). The ration analysis was done using 50 seeds for each event and was used as indication of a single copy number of the transgenic gene. Total DNA from regenerated heterozygous plants was extracted using the Wizard® Genomic DNA Purification kit (Promega - Madison, Wisconsin, USA), following the manufacturer's instructions. Twenty events were used for pathogenicity testing with *C. graminicola*. To evaluate *CHSVII* gene silencing in *C. graminicola* and the expression of the *bar* gene in transgenic plants, total RNA was extracted from leaves of inoculated plants with *C. graminicola*. RNA extraction was performed using the SV Total RNA Isolation System kit (Promega, Madison, Wisconsin, USA), following the manufacturer's recommendations. Leaf samples from hybrid non-transgenic HiII/L3 plants at the same phenological stage as the transgenic plants were used as negative controls. For cDNA synthesis, 1.0 μg of total RNA, previously treated with DNase I, was used with the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific - Waltham, Massachusetts, USA). The reverse transcription reaction was carried out under the following conditions: 25 °C for 10 min, 37 °C for 2 h, and 80 °C for 5 min. For the PCR reactions, the cDNA was diluted 1:10 and added to the reaction mix, which consisted of 5.0 μL of Fast SYBR® Green Master Mix (Thermo Fisher Scientific - Waltham, Massachusetts, USA), 0.25 μL of each primer (10 μM) and 1.5 μL of water treated with DEPC (Diethyl Pyrocarbonate) treated water. Reactions were performed on QuantStudio 6 – 7500 Fast RealTime PCR System (Thermo Fisher Scientific - Waltham, Massachusetts, USA). Primers used to quantify the expression of the *bar*, *CHSVII* and *actin* (control) genes are listed in Table S1. All experiments were conducted in biological and technical triplicate, and relative gene expression levels were calculated by the $2_{\bar{T}}^{-\Delta\Delta C}$ [43].

2.7. Transgenic plants inoculation assay

Conidial suspensions were prepared from the monosporic *C. graminicola* isolate 10.09 in Oat Flour Agar media under continuous light for 7-8 days. Sporulation was induced by scrapping the mycelium after 4 days and after 5-6 days the isolates were transferred to Petri dishes containing Oat Flour Agar. After an additional 5 five days the plates were flooded with distilled water and scraped to release the conidia, which were counted using a Neubauer chamber and diluted to a final concentration of 10^6 conidia/mL.

C. graminicola inoculation was performed on 30-day-old transgenic maize plants grown in 20 L pots by spraying a conidial suspension at concentration of 10^6 conidia/mL. Each event tested 8 plants divided into two pots. After four weeks, the fourth leaf of each plant was collected and photographed alongside a reference scale to enable image-based analysis. Six days later, stalks were inoculated using toothpicks embedded in *C. graminicola* suspension containing 10^6 conidia/mL. Finally, 16 days post inoculation, stalks and leaves were harvested and

photographed. Anthracnose severity assessments were performed at 0 and 18 days after the first application, using a rating scale from 0 to 5 (0 = absence of leaf lesions and 5 = lesions on 100% of the leaves, with plant drying) [44].

2.8. Statistical analysis

All statistical analyses were performed using R software, version 4.3.2 [45]. Disease severity was quantified using the pliman package [46], which enables automated image analysis of maize leaves and stalks. Assumptions of the linear models, including normality of residuals and homogeneity of variances, were assessed using functions from the performance package [47]. When model assumptions were satisfied, an analysis of variance (ANOVA) was performed, followed by estimated marginal means with multiple comparisons adjusted by Tukey's test using the emmeans package (https://cran.r-project.org/web/packages/emmeans/index.html). All graphical visualizations were generated using the ggplot2 package (https://cran.r-project.org/web/packages/ggplot2/index.html). A significant level of p-value <0.05 was adopted for all statistical tests.

3. Results

3.1. RNAi machinery in C. graminicola is conserved and functional

Key genes associated with the RNA silencing machinery have been identified in the *C. graminicola* genome. These loci encode three RNA-dependent RNA polymerase (RDR) homologs (*RDR1*, *RDR2*, and *RDR3*), two Dicer homologs (*DCL1* and *DCL2*), and two Argonaute homologs (*AGO1* and *AGO2*). Phylogenetic analysis of the proteins encoded by these genes identified in *C. graminicola* and several *Ascomycota* species allowed them to be clustered with components of the Quelling or MSUD (meiotic silencing by unpaired DNA) pathways, previously described in other well-characterized fungal species. In addition to *C. graminicola*, ten other *Colletotrichum* species also exhibit conservation of these critical components, which are essential for effective RNA silencing in fungi (Fig. 1). The RDR2 protein of *C. graminicola* diverged from both the Quelling and MSUD groups, clustering instead with the orphan RDR3 from *Neurospora crassa*, whose function remains unclear (Fig. 1) [39].

Proteins from the RDR, DCL, and AGO families across the eleven *Colletotrichum* species formed a homogeneous clade with strong bootstrap values above 90%, indicating a high degree of conservation within the genus (Fig. 1). All genes associated with distinct RNA silencing pathways identified in the *C. graminicola* genome are intact, and the corresponding pathways have the minimal set required for functionality. These genes may be expressed during the asexual and/or sexual stages of the fungal life cycle, assuming that the silencing machinery is functionally active. However, the Quelling pathway is likely the primary contributor, as species within the *Colletotrichum* genus are typically in the anamorphic (asexual) stage during pathogenesis, when they induce anthracnose symptoms in plants [48].

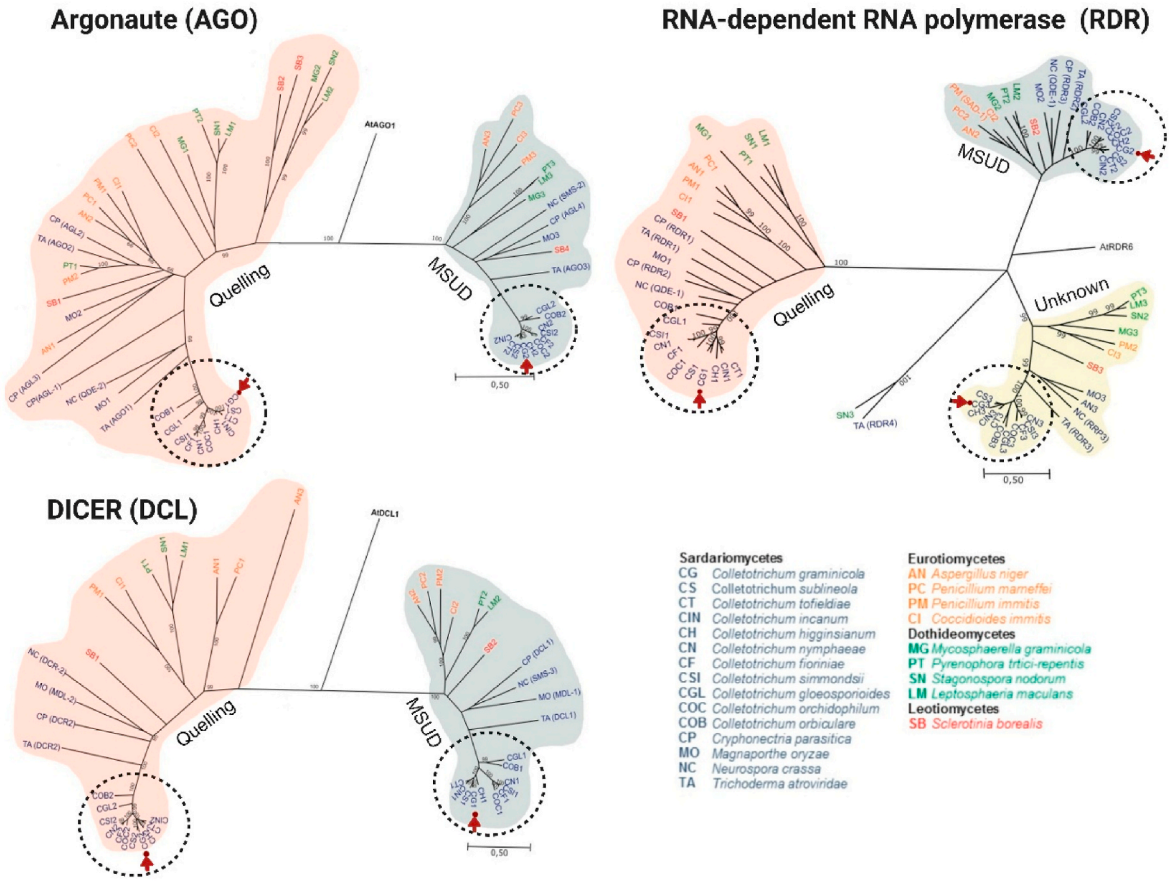


Fig. 1. RNA silencing pathways in the Ascomycota fungus *Colletotrichum graminicola*. Phylogenetic analysis of key protein sequences of the RNA silencing pathway, Argonauta (AGO); Dicer-like (DCL); RNA-dependent RNA polymerase (RDR). Phylogenetic trees of representative members of the Ascomycota clade (*Sordariomycetes* in blue shaded area, *Eurotiomycetes* in orange shaded area, *Dothideomycetes* in green shaded area and *Leotiomycetes* in red shaded area. Proteins from *Colletotrichum* spp. subclade (containing the proteins of *Colletotrichum higginsianum* that possess functional RNAi machinery) and *C. graminicola* are highlighted with a dashed circle *and* a red arrow, respectively. Two main groups were marked, the "Quelling pathway" and the "Meiotic-Silencing by Unpaired DNA (MSUD) pathway". (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

To investigate whether RNA silencing pathways are active *C. graminicola*, fungal cultures were treated with 1 μg of siRNAs derived from the *C. graminicola* CHSV target (*siCHSV*). siRNAs are reported as interesting molecular triggers for gene silencing in several fungal species [49] (Fig. 2A). When comparing the expression of genes from each silencing pathway (Quelling, MSUD, and Unknown) in mycelia exposed to *siCHSV*, a significant upregulation of genes associated with Quelling (AGO1, DCL1, and RDR3) was observed, relative to the expression levels of components from the other pathways (Fig. 2A). Although genes associated with the MSUD (AGO2, DCL2, and RDR1) and the Unknown pathway (RDR2) were also expressed, their expression levels were much lower, which supports the fact that the asexual stage of *C. graminicola* is active in this context [48] (Fig. 2A).

Biological responses in knockdown of *CHSV*, *CHSVII* and *PPT* expressions were also validated, when siRNAs and dsRNAs were used compared to the control treatment derived from GFP (*siGFP* and *dsGFP*). This reduction in transcript accumulation indicates that exposure to target-specific siRNAs and dsRNAs triggers an effective RNA silencing response.

Gene expression analysis by RT-qPCR showed that *C. graminicola,* cultivated on solid medium in the presence of siRNA (*siPPT, siCHSV*, and *siCHSVII*), exhibited a significant reduction in transcript levels for all tested genes. This effect was not observed in treatments with dsRNA (*dsPPT1, dsCHSV*, and *dsCHSVII*), where the transcripts levels remained statistically unchanged for all genes in comparison to the control (*dsGFP*) (Fig. 2B). The siRNA treatments were grouped by the Tukey test ($p < 0.05$). Among the siRNA treatments, *C. graminicola* exposed to siPPT showed the highest reduction in transcript levels, with a 60% decrease in the number of transcripts compared to *siGFP*. The *siCHSV* and *siCHSVII* treatments achieved 46% and 53% reductions in the number of transcripts, respectively (Fig. 2B).

The results obtained regarding the mycelial dry biomass analysis are consistent with the gene expression data. A significant reduction in mycelial biomass was observed in siRNA treatments, while treatments with dsRNA did not result in significant biomass reduction (Fig. 2C). These results may be associated with the inefficiency in cellular absorption and transport of dsRNA molecules from extracellular to intracellular compartments biomass via endocytosis [50]. Among the siRNA treatments, *siPPT* and *siCHSV* showed higher reductions in dry biomass (75% compared to the GFP control), whereas the *siCHSVII* treatment led to a reduction of around 45% (Fig. 2C). In the ANOVA test (Tukey $p < 0.05$), *siPPT* and *siCHSV* were grouped together, while the *siCHSVII* formed a separate group; nonetheless, all siRNA treatments showed statistically significant differences compared to the control.

3.2. Silencing efficiency of C. graminicola target genes saturates at 1.0 μg siRNA

The results of the *in vitro* assays to evaluate the most efficient dose-response for inducing gene silencing, using 0.5, 1.0, or 2.0 μg of siRNA, yielded similar results across the different target genes. For the *PPT (siPPT)* and *CHSV* (*siCHSV*) genes, the highest dose (2 μg) showed statistically the same efficiency in reducing transcripts, although there was a clear trend of silencing ineffectiveness when exceeding the 1.0 μg dose (Fig. 3). The concentration of 1 μg, for most genes, showed the most consistent results for future assays, with an estimated reduction of 50% for the *PPT* gene and 80% for *CHSV*. For the *CHSVII (siCHSVII)* gene, both 1.0 μg and 2.0 μg siRNA resulted in a gene expression reduction between 40% and 50%. Although the graph shows a trend of increased silencing efficiency at the highest concentration (2.0 μg), no statistical difference was observed (Fig. 3). A similar pattern has been observed in other biological systems, where excessively high siRNA concentrations result in diminished silencing efficiency. This may be associated with the saturation of proteins involved in the silence pathway, which, due to the accumulation of molecules, may become unable to completely process them [19]. These saturation effects have already been reported in studies with insects, flatworms, and the fungus *Sclerotinia sclerotiorum*, where high doses of dsRNA were less effective than intermediate doses

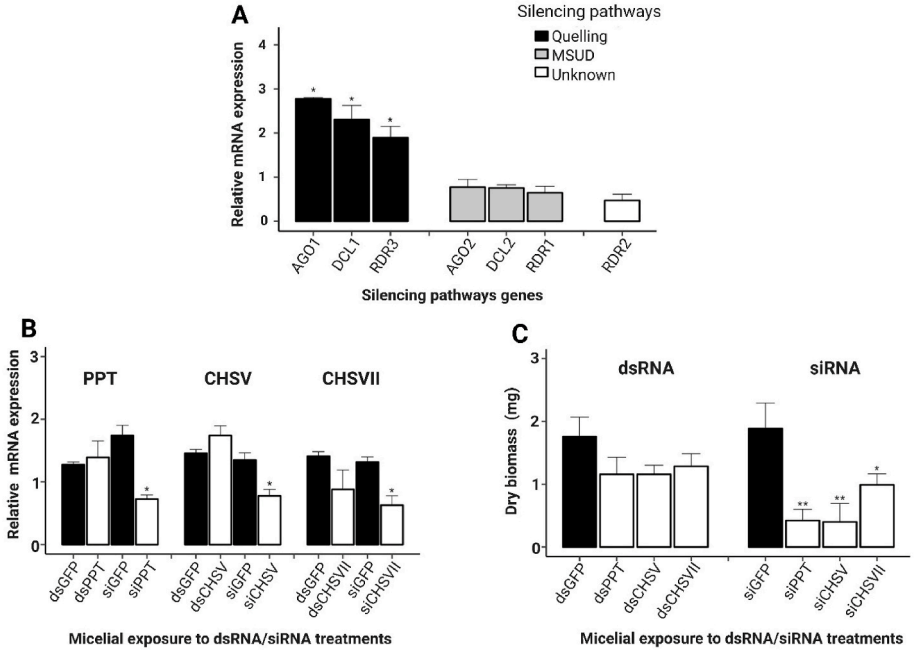


Fig. 2. Induction of RNA silencing pathways in *Colletotrichum graminicola*. Expression of key genes of distinct silencing pathways in *C. graminicola* of the classes Argonaute (AGO), Dicer-like (DCL), and RNA-dependent RNA polymerase (RDR) during mycelial exposure to siCHSV. Genes are grouped according to their respective originating pathways "Quelling", MSUD, and "Unknown" **(A).** Induction of RNA silencing in *C. graminicola in vitro* using siRNAs and dsRNAs. Relative quantification of *PPT, CHSV* and *CHSVII* gene expression in *C. graminicola* mycelium grown on Potato Dextrose agar exposed to treatment with dsRNA (~300bp)/siRNA (~20bp) molecules derived from the respective dsPPT/siPPT, dsCHSV/siCHSV and dsCHSVII/siCHSVII genes **(B).** Quantification of dry biomass (mg) of *C. graminicola* mycelium grown in liquid Potato Dextrose broth under continuous exposure to dilutions of dsRNA/siRNA derived from the *PPT*, *CHSV* and *CHSVII* genes of *C. graminicola* **(C).**

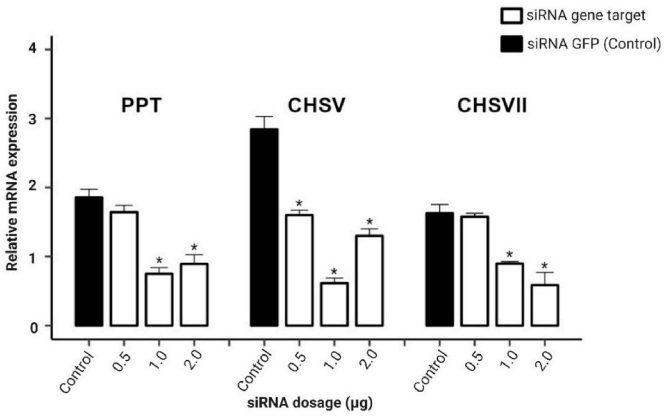


Fig. 3. Comparative analysis of siRNA dose-response in *Colletotrichum graminicola in vitro*. The replicates of *C. graminicola* grown on a solid PDA medium and exposure to siRNAs (~20 bp) of *PPT* (siPPT), *CHSV* (siCHSV), and *CHSVII* (siCHSVII), genes at different concentrations: 0.5 µg; 1.0 µg, and 2.0 µg. The lowest values of relative quantification were considered the best responses to gene silencing.

in triggering gene silencing [19,51–53].

3.3. Spray-induced gene silencing response to dsRNAs and siRNAs in maize leaves

The results observed in the maize leaf (BRS1010) inoculated with *C. graminicola* conidia exposed to dsRNA and siRNA molecules (Fig. 4) were opposite to those observed in culture medium, considering the effectiveness of both molecules in suppressing *C. graminicola* gene expression pattern of the target genes. Extensive necrotic lesions observed on leaves, treated with siRNA, showed higher percentage of infection of *C. graminicola* compared to the dsRNA treatments (Fig. 4A) indicating lower effectiveness of the siRNA molecules to control *C. graminicola* under this condition. The treatment of siRNA showed no statistically significant difference compared to *siGFP* control. The lesion extension was slightly greater, however lesions progression in the treatments was similar. Interestingly, as previously mentioned, the topical application of dsRNA to maize leaves was effective in protecting against *C. graminicola* infection (white dashed circles - Fig. 4A), even though dsRNA was not the most effective treatment for inducing silencing in *C. graminicola* under *in vitro* conditions. A significant reduction in lesion size was observed in the *dsPPT* and *dsCHSVII* treatments compared to the *dsGFP* control, with anthracnose severity reduced by approximately 85%. Although the *dsCHSV* treatment did not show a statistically significant difference in lesion size, it exhibited a trend toward reduced severity (Fig. 4B).

All treatments with dsRNA (*dsPPT, dsCHSV*, and *dsCHSVII*) resulted in a significant reduction in transcripts levels, whereas all siRNA treatments (*siPPT, siCHSV*, *siCHSVII*, and *siPPT*) showed no significant differences in transcripts levels compared to the control (Fig. 4C). The data demonstrates a higher efficiency of dsRNA molecules compared to siRNAs inducing gene silencing via foliar application. The treatment with the greatest reduction in transcripts was observed for *dsCHSVII* (85%), while *dsPPT* and *dsCHSV* showed reductions of 80% and 40%, respectively. According to the Tukey test ($p < 0.05$), all siRNA treatments (*siPPT1, siCHSV,* and *siCHSVII*) were grouped as statistically equal

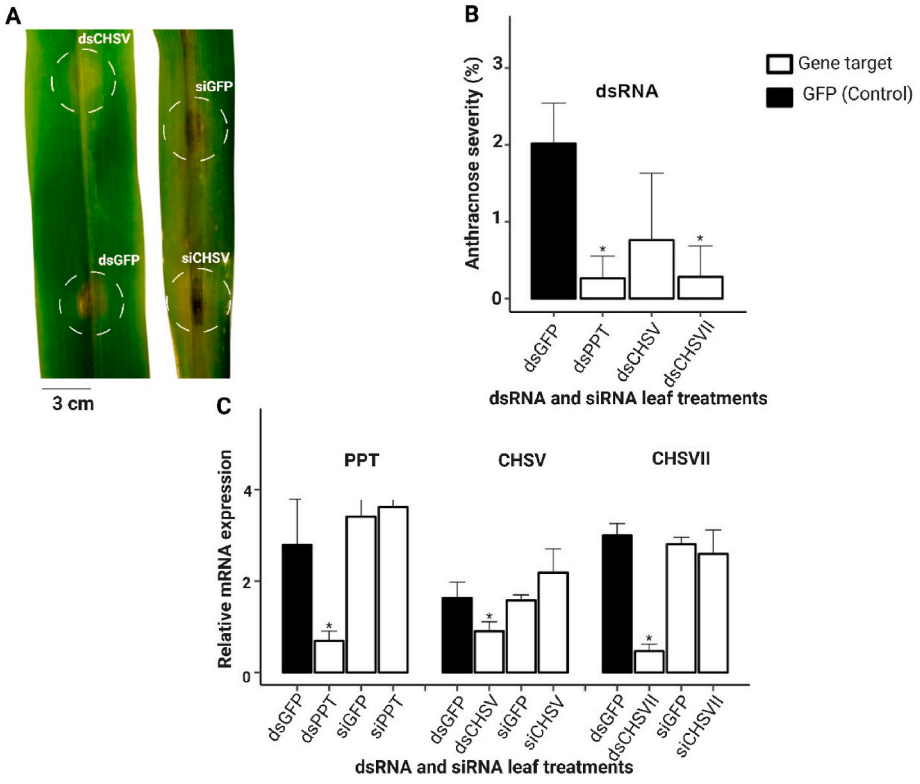


Fig. 4. Effect of application of dsRNAs to siRNAs on the severity of anthracnose in maize caused by *Colletotrichum graminicola*. Necrotic lesions caused by *C. graminicola* in experimental triplicates of maize leaves (BRS1010) after exposure to 1 µg of dsRNA (dsPPT, dsCHSV and dsCHSVII) and siRNA (siPPT, siCHSV and siCHSVII) derived from the *PPT*, *CHSV* and *CHSVII* genes of *C. graminicola*. White dashed circles indicate where ds/siRNAs were applied with *C. graminicola* spores **(A).** Severity of anthracnose in detached leaves after the treatment with dsRNA molecules from the *PPT*, *CHSV* and *CHSVII* genes of *C. graminicola* (seven days after inoculation) **(B).** Relative quantification of the expression of *PPT*, CHSV and *CHSVII* genes in maize leaves (BRS1010) inoculated with *C. graminicola* treated with dsRNA (dsPPT, dsCHSV and dsCHSVII) and/or siRNA (siPPT, siCHSV and siCHSVII) derived from the *PPT*, *CHSV* and *CHSVII* genes of *C. graminicola* (seven days after inoculation) **(C).**

(Fig. 4C).

3.4. Transgenic maize lines expressing dsRNA are resistant to C. graminicola

The RNAi construct contains 311 bp fragments of the *CHSVII* gene from *C. graminicola* in a duplicated inverted orientation, interspersed by an intron sequence (Fig. 5A). This gene construction when transcribed forms dsRNA. The expression of the *CHSVII* dsRNA (*dsCHSVII*) is controlled by the Ubiquitin promoter, with glufosinate selection (bar gene). The integration of the T-DNA containing the transgenic gene in the maize plants was confirmed by PCR amplification of the *bar* and target genes sequences. Positive plants exhibited a 502 bp amplicon for the bar gene, whereas the non-transformed parental HiII and L3 plants, used as negative controls, showed the expected negative result (Fig. 5B).

Progenies of 20 events confirmed to contain a single copy of the *CHSVII* RNAi construct and in heterozygous state were tested against an artificial infestation of *C. graminicola* in the maize GMO events. The results were based on a classification from 0 to 5 grade based on the severity of the disease (0 no effect and 5 the plant is dead) and showed a gradient from low to high severity effect (Fig. 5C).

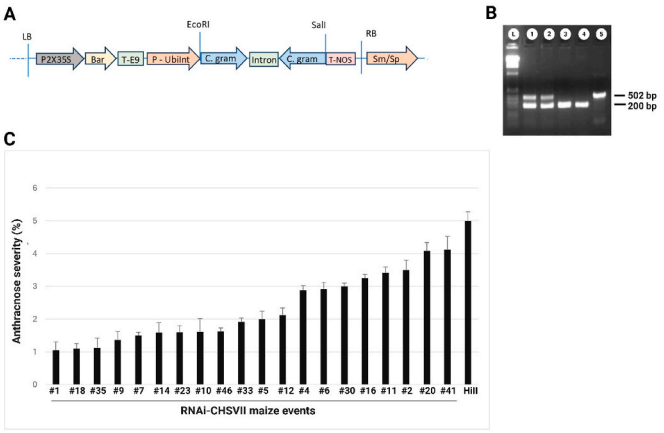


Fig. 5. – Gene construct for CHSVII dsRNA, PCR for presence of the gene construct in maize transgenic plants and comparison of the effect of anthracnose severity on the different maize transgenic events evaluated under the same conditions. Schematic representation of the gene constructs used in maize transformation. LB = left border repeat of the octopine Ach5 T-DNA; RB = right border repeat of nopaline C58 T-DNA; P-UbiInt = maize Ubiquitin promoter modified with the second intron of the potato ST-LS1 gene (Vancanneyt et al., 1990); NOS-T = Nos terminator; Sm/Sp' = aadA gene/aminoglycoside 3′-adenyltransferase gene from Shigella flexneris 2a, which confers resistance to the antibiotic spectinomycin and streptomycin, naturally found in the B819 vector (Murphy 1985) **(A)**. PCR confirmation of T-DNA integration from p7i2XUbiZm construct into one of 20 transgenic maize plants. Transgenic plants were heterozygous state since has been crossed with non-transgenic lines. The copy number is deduced by segregation ratio of the bar gene when plants were sprayed with the herbicide ammonia glufosinate (Finale). PCR products were amplified using primers for *Bar* gene (~502 bp) and primers for *actin* gene (~200 bp) (Table S1) and loaded onto agarose gel (2.5%) stained with GelRed. L-1Kb DNA Ladder; Samples of DNA of the leaves from maize plants: RNAi-control (Hi-II x L3) hydrid (1); RNAi-CHSVII event #1 (Hi-II x L3) hydrid (2); Hi-II (parental wild type 1) (3), L3 (parental wild type 2) (4) and plasmid p7i2XUbiZm (5) **(B).** The RNAi-control plants are also hybrid that shares the same parental lineage as RNAi-CHSVII but differently expresses a hairpin of a target gene derived from *Puccinia polysora*, conserved only in *Pucciniaceae*, with no homology to *C. graminicola* in the same T-DNA backbone. The samples 3 and 4 (negative control; non-transgenic parental lines) and 5 (positive control; plasmid used to transform maize plants). Effect of 20 transgenic maize events containing the CHSVII dsRNA gene construct. Each treatment used 2 pots with 4 plants each. Plants F1 were inoculated with *C. graminicola* and the severity graded from 0 to 5 (0 = no effect and 5 plant is dead) after 14 days. Plants were heterozygous and single copy transgenic gene **(C).**

Disease severity in RNAi-CHSVII stalks and leaves showed a significant reduction on transgenic maize plants compared to the other not containing the same gene construction. The reduction was about 30% lower compared to the RNAi-control (Fig. 6A), and compared to the results presented in the *in vitro* and *in vivo* assay supporting the idea that the *CHSVII* genes as potential targets for protection against *C. graminicola*. It is possible that combining a broader set of host-derived target genes could improve the response of transgenic plants to phytopathogenic fungi.

4. Discussion

Eukaryotic sRNAs are generally categorized into three main classes: short interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-interacting RNAs (piRNAs). RNA mediates silencing mechanisms, as a transcriptional and post transcriptional level in eukaryote cells [54], involves formation of double-stranded RNA (dsRNA) that is processed into small RNAs (21–30-nucleotide) by the ribonuclease III like enzyme Dicer or Dicer-like (DCL) [55]. The small RNAs are incorporated into an RNA-induced silencing complex (RISC) that contains an argonaute (AGO) member of protein family [56]. In many organisms, RNA-dependent RNA polymerases (RdRPs), another component of the RNAi mechanism, are capable to amplify sRNA signals by generating dsRNA from single stranded RNA templates [57–59]. RdRP activity was detected in the tissue of numerous plants that were thought to be uninfected [60,61]. Subsequently, it was shown that the plant RdRP gene had homologs in fungi [quelling in *N. crassa* in *Neurospora crassa* [58]. Members of the DCL, AGO, and RDR protein families from Ascomycota fungi, known to participate in different silencing pathways, are clustered into three groups: "Quelling," "MSUD," and "Unknown". The "quelling" mechanism comprises a post-transcriptional gene silencing (PTGS) during *N. crassa* vegetative (asexual) cycle and is responsible for recognizing repetitive DNA templates and therefore silencing its homologous genes [62]. Meanwhile, the MSUD (Meiotic Silencing of Unpaired DNA) occurs during the sexual cycle, in Meiosis I prophase, and leads to the silencing of matching sequences of unpaired DNA sequences between the two parental chromatids [63–65]. In this study it was correlated the RNA silencing machinery genes in the phytopathogen ascomycete fungus *C. graminicola*, how these genes respond to siRNA and dsRNA and how this response affects the growth in culture media and severity of the disease anthracnose in the maize plant.

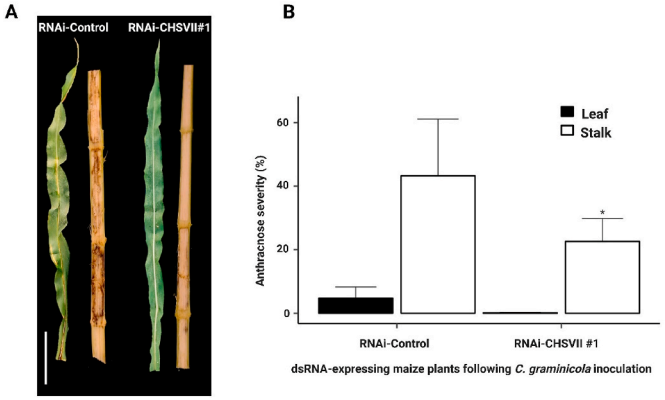


Fig. 6. – Host-induced gene silencing of the *CHSVII* hairpin derived from *Colletotrichum graminicola* in transgenic maize plants on the severity of anthracnose caused by *C. graminicola*. Symptoms of severity of anthracnose on maize stalk and leaves in hybrid transgenic plants expressing the *C. graminicola CHSVII* hairpin in the RNAi-control maize event and RNAi-CHSVII event #1 (A). Quantification of anthracnose severity in maize stalk and leaves in hybrid transgenic plants expressing the *C. graminicola CHSVII* hairpin in the RNAi-control maize event and RNAi-CHSVII event #1 (B).

The observed difference in gene silencing efficiency between dsRNA and siRNA treatments in *C. graminicola* cultivated in media may be related to differential cellular uptake capacity in fungi. Factors associated with fragment size and cell transport mechanisms have been evaluated in studies with *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, and *Candida albicans*. These studies investigated the RNA fragments mobility occur via endocytosis and that the vesicular content consists of molecules smaller than 250 bp [50]. Due to the limited capacity of extracellular vesicles to transport larger molecules, it is likely that dsRNA molecules were not mobilized to the cytoplasm and, therefore, did not induce gene silencing. In contrast, siRNAs were likely mobilized into the cytoplasm, activating the degradation of target transcripts. Other studies with *Aspergillus nidulans* highlighted the efficiency of siRNA in inducing gene silencing *in vitro* assays [20,49].

A lower efficiency observed in siRNA treatments may be due to the limited ability of siRNA molecules to penetrate plant cells via foliar application. A study on gene silencing induction through the application of siRNA in *Nicotiana benthamiana* also showed the ineffectiveness of siRNA molecules inducing gene silencing through foliar applications. Effective silencing was only achieved through the application of siRNA via high-pressure spray, which enabled direct penetration of the molecules into the cytoplasm [66]. In contrast, in all dsRNA treatments with (*dsPPT, dsCHSV*, and *dsCHSVII*), *C. graminicola* did not successfully colonize following foliar application. These results agree with the gene expression data, confirming that gene silencing through dsRNA application occurred effectively. Another relevant aspect is that even in areas treated with *dsGFP,* disease symptoms did not progress. This can be understood as the induction of silencing in dsRNA treatments possibly activating systemic propagation through intracellular movement. Similar findings have been reported in other studies involving fungi and aphids, in which foliar application of dsRNA was effective, showing that leaves can absorb dsRNA molecules and can activate silencing through the plant's intrinsic silencing RNAi machinery [19,21,67].

One of the most challenges to use RNAi mechanism to inhibit gene expression is the propensity of siRNAs/dsRNAs knock down the expression of other genes decides the desired target. These off-target effects can occur when the siRNA is partially complementary to one or more cellular mRNAs (besides the target) causing degradation [68]. In this work no significant identity to *C. graminicola* dsRNA (*CHSV, CHSVII and PPT1*) was found to the maize genome (*e.g.*, B73 RefGen_v4) using BLAST (NCBI). Other specialized off-target search software such as dsCheck (https://dscheck.rnai.jp/), does not have the search in the maize and *C. graminicola* genomes.

Spray-induced gene silencing has demonstrated efficacy in laboratory settings against various fungal pathogens through a technology called spray-induced gene silencing (SIGS). Although it is non-transgenic and environmentally friendly, it might be an alternative to chemical fungicides. Research progressed from the laboratory to the greenhouse and field environments, novel challenges arose, such as ensuring the stability of dsRNAs and their effective delivery to fungal targets [69].

To test a hypothesis of gene function in transgenic plants, researchers must consider a wide range of factors spanning experimental design, molecular characterization, and phenotypic analysis. In this case it is a gain-of-function (overexpression a dsRNA molecule) and its relation to severity of anthracnose disease caused by *C. graminicola* infection. A sensitive hybrid (HiII) was used to be transformed with a gene constructed with the sequence of the *CHSVII* gene from *C. graminicola* cloned duplicated in inverted position in a binary vector suitable for maize transformation. This gene construct once is transcribed makes a double strand RNA based on snapping the homologue sequence created on the duplicated inverted sequence. These molecules can activate the RNAi machinery in the maize plant and in the *C. graminicola* and consequently processed into small pieces until reach to the 19-23 pb (siRNAs). The time required to be processed, and the amount of dsRNA and siRNA depends on many factors that are not been considered in this paper. Each transgenic event plants varies the expression of the gene. However, to have a representative event of highly expressed genes is required to generate at least 30-50 events. Some events that contain more than one copy or are incapable of generating progeny are eliminated in the F1 population. In this word we have end up with 20 events out of 30 that are single copy gene and generated F1 seeds. The F1 are heterozygous plants since they came from T0 plants crossed with a non-transgenic line (L3). The expected ration of resistant to nonresistant individuals is 1:1 for a single copy gene.

The inhibition of phytopathogenic fungi by dsRNA expressed in transgenic plants has been demonstrated for several fungi species, such as *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Aspergillus oryzae*, *Aspergillus niger*, and *Magnaporthe oryzae* [70]. Regarding transgenic plants, maize plants expressing RNAi constructs were successful in reducing *Aspergillus flavus* infection, consequently leading to lower aflatoxin synthesis. This clearly demonstrates the efficacy of RNAi-based strategies as an alternative control technology [71,72].

These results are expected since the insertion of the gene construct at different location in the maize genome might cause difference in the expression of the dsRNA. The correlation of the higher expression of CHSVII dsRNA to the phenotypic data of anthracnose resistant maize transgenic events has not been analyzed although it is solid evidence of the effect of the dsRNA to the phenotype. According to the phenotypic data there are more than 50% of the transgenic maize events with severity below 2 in a scale until 5 and even for the most affected events the severity is below the non-GMO control (maize plant HiII) indicating the effect of the CHSVII dsRNA. RNAi-CHSVII plants clearly showed reduced severity in stalks tissues. Besides that, the leaves of RNAi-*CHSVII* plants consistently showed a more vibrant green color and a complete absence of necrosis in the central vein after inoculation with *C. graminicola*.

5. Conclusions

The plant pathogen *C. graminicola*, which causes anthracnose, can be controlled by RNAi mechanism in maize. In this study, in the dose–response assays with siRNA, it was found that higher concentrations may be ineffective at inducing gene silencing, possible due to the saturation of enzymes involved in the RNA silencing pathway. Thus, an intermediate concentration (1 μg) yielded the most effective silence results. Transcripts levels were reduced *in vitro* assays with *C. graminicola*, in treatments involving spores exposed to siRNA (*siPPT1, siCHSV*, and *siCHSVII*), demonstrating greater efficiency for fungi in capturing smaller molecules via the endocytic pathway. Regarding the results for mycelial dry biomass, these corroborated with the gene expression data, with siRNA treatments (*siPPT, siCHSV*, and *siCHSVII*) showing the lowest mycelial weight in liquid medium. Conversely, the assays based on foliar application in maize showed that dsRNA promoted effective gene silencing, a fact verified by the reduction of gene expression and confirmed by the considerable reduction in necrotic lesions of the inoculated leaves and stalks. Parameters of spraying dsRNA at larger scale in the field such as price of kg of dsRNA and stability of the molecules in the field are still under investigation. However, the possibility of expressing dsRNA in transgenic maize lines is quite feasible as shown on the positive results obtained in this work. Transgenic maize lines expressing dsRNA have shown effectiveness both in leaves and stalks against *C. graminicola* infection using the CHSVII gene. These findings indicate that the use of dsRNA provided effective protection against *C. graminicola* infection either by topical application or over expressing dsRNA in transgenic lines. The results of the present study are consistent with previous reports presented in the literature, particularly those involving dsRNA applications in plants and siRNA use *in vitro* systems [19–21,66].

CRediT authorship contribution statement

Raquel Pereira Passos Salgado: Investigation, Data curation. **Gabriel Angelo Saraiva Raimundo:** Methodology, Investigation, Conceptualization. **Lucas Romão Gava:** Validation, Methodology. **Andréa Almeida Carneiro:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology. **Fernando Hercos Valicente:** Writing – review & editing. **Dagma Dionísia da Silva:** Methodology. **Luciano Viana Cota:** Methodology. **Meire de Cássia Alves:** Methodology. **André da Silva Xavier:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation. **Newton Portilho Carneiro:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Formal analysis.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to correct grammar/English structure. After using this tool/service, the author(s) reviewed and edited the content as needed and take (s) full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thanks the Fundação de Amparo a Pesquisa do Estado de Minas Gerais (Fapemig - APQ-02674-18) and the Empresa Brasileira de Pesquisa Agropecuaria (Embrapa) for the financial and institutional support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2026.103161.

Data availability

Data will be made available on request.

References

[1] M.C. Fisher, N.J. Hawkins, D. Sanglard, S.J. Gurr, Worldwide emergence of resistance to antifungal drugs challenges human health and food security, Science 360 (6390) (2018) 739–742, https://doi.org/10.1126/science.aap7999.
[2] L. Chang, Y. Li, Z. Gao, P. Bonello, M. Cleary, I.A. Munck, A. Santini, H. Sun, Novel pathogen–plant host interaction: *Colletotrichum jiangxiense* and *Fraxinus americana* L. (white ash) in a sentinel garden in China, Plants 12 (23) (2023) 4001, https://doi.org/10.3390/plants12234001.
[3] V. Guarnaccia, J.Z. Groenewald, G. Polizzi, P. W Crous, High species diversity in *Colletotrichum* associated with citrus diseases in Europe, Persoonia-Molecular Phylogeny and Evolution of Fungi 39 (1) (2017) 32–50, https://doi.org/10.3767/persoonia.2017.39.02.
[4] Y.C. Sun, U. Damm, C.J. Huang, *Colletotrichum plurivorum*, the causal agent of anthracnose fruit rot of papaya in Taiwan, Plant Dis. 103 (5) (2019) 1040, https://doi.org/10.1094/PDIS-08-18-1423-PDN.
[5] C. Bellé, R. F Ramos, R. Moccellin, C.R. J de Farias, Detection of *Colletotrichum coccodes* causing leaf anthracnose on *Pisum sativum* in southern Brazil, J. Plant Pathol. 102 (1) (2020), https://doi.org/10.1007/s42161-019-00392-6, 255-255.
[6] M. Guevara-Suarez, M. Cárdenas, P. Jiménez, L. Afanador-Kafuri, S. Restrepo, *Colletotrichum* species complexes associated with crops in Northern South America: a review, Agronomy 12 (3) (2022) 548, https://doi.org/10.3390/agronomy12030548.
[7] H. Ma, L. Huang, L. Guo, S. Chen, J. Liu, C. Liu, D. Yanxia, S. Xianchao, H. Lin, G. Ma, Identification and management of a novel Danshen leaf anthracnose caused by *Colletotrichum karstii* in *Salvia miltiorrhiza* Bunge in China, Front. Plant Sci. 16 (2025) 1526038, https://doi.org/10.3389/fpls.2025.1526038.
[8] Y. Qian, X. Wang, X. Zhai, X. Hu, T. Li, Y. Li, Q. Xiong, First report of anthracnose caused by *Colletotrichum gloeosporioides* on lucky bamboo in China, Forests 16 (1) (2025) 128, https://doi.org/10.3390/f16010128.
[9] R.V. da Costa, L.V. Cota, D.D. da Silva, D.F. Parreira, C.R. Casela, E.C. Landau, J.E. F. Figueiredo, Raças de *Colletotrichum graminicola* patogênicas ao milho no Brasil, Embrapa Milho e Sorgo. Boletim de Pesquisa e Desenvolvimento 96 (2014) 31p.
[10] D.D. de Silva, P.W. Crous, P.K. Ades, K.D. Hyde, P.W.J. Taylor, Life styles of *Colletotrichum* species and implications for plant biosecurity, Fungal Biology Reviews 31 (3) (2017) 155–168, https://doi.org/10.1016/j.fbr.2017.05.001.
[11] E.S. Jacobson, R. Ikeda, Effect of melanization upon porosity of the cryptococcal cell wall, Med. Mycol. 43 (4) (2005) 327–333, https://doi.org/10.1080/13693780412331271081.
[12] J.A.L. van Kan, Licensed to kill: the lifestyle of a necrotrophic plant pathogen, Trends Plant Sci. 11 (5) (2006) 247–253, https://doi.org/10.1016/j.tplants.2006.03.005.
[13] A. Nicoli, L. Zambolim, R.V. da Costa, L.J.M. Guimarães, F.E. Lanza, D.D. da Silva, L.V. Cota, Identification of sources of resistance to anthracnose stalk rot in maize, Ciência Rural. 46 (11) (2016) 1885–1890, https://doi.org/10.1590/0103-8478cr20151052.
[14] R.C. Wilson, J.A. Doudna, Molecular mechanisms of RNA interference, Annu. Rev. Biophys. 42 (2013) 217–239, https://doi.org/10.1146/annurev-biophys-083012-130404.
[15] J.M. Villalobos-Escobedo, A. Herrera-Estrella, N. Carreras-Villaseñor, The interaction of fungi with the environment orchestrated by RNAi, Mycologia 108 (3) (2016) 556–571, https://doi.org/10.3852/15-246.
[16] A. Younis, M.I. Siddique, C.K. Kim, K.B. Lim, RNA interference (RNAi) induced gene silencing: a promising approach of hi-tech plant breeding, Int. J. Biol. Sci. 10 (10) (2014) 1150, https://doi.org/10.7150/ijbs.10452.
[17] S.B. Ghag, Host induced gene silencing, an emerging science to engineer crop resistance against harmful plant pathogens, Physiol. Mol. Plant Pathol. 100 (2017) 242–254, https://doi.org/10.1016/j.pmpp.2017.10.003.
[18] C.C. Nunes, R.A. Dean, Host-induced gene silencing: a tool for understanding fungal host interaction and for developing novel disease control strategies, Mol. Plant Pathol. 13 (5) (2011) 519–529, https://doi.org/10.1111/j.1364-3703.2011.00766.x.
[19] A.G. McLoughlin, N. Wytinck, P.L. Walker, I.J. Girard, K.Y. Rashid, T. de Kievit, W. G.D. Fernando, S. Whyard, M.F. Belmonte, Identification and application of exogenous dsRNA confer plant protection against *Sclerotinia sclerotiorum* and *Botrytis cinerea*, Sci. Rep. 8 (1) (2018) 7320, https://doi.org/10.1038/s41598-018-25434-4.
[20] M. Khatri, M.V. Rajam, Targeting polyamines of *Aspergillus nidulans* by siRNA specific to fungal ornithine decarboxylase gene, Med. Mycol. 45 (3) (2007) 211–220, https://doi.org/10.1080/13693780601158779.
[21] A. Koch, D. Biedenkopf, A. Furch, L. Weber, O. Rossbach, E. Abdellated, L. Linicus, J. Johfnnsmeier, L. Jelonex, A. Goesmann, V. Cardoza, J. McMillan, T. Mentzeil, K. H. Kogel, RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs, PLoS Pathog. 12 (2016) e1005901, https://doi.org/10.1371/journal.ppat.1005901.
[22] D. Nowara, A. Gay, C. Lacomme, J. Shaw, C. Ridout, D. Douchkov, P. Schweizer, HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*, Plant Cell 22 (9) (2010) 3130–3141, https://doi.org/10.1105/tpc.110.077040.
[23] V. Panwar, B. McCallum, G. Bakkeren, Host-induced gene silencing of wheat leaf rust fungus *Puccinia triticina* pathogenicity genes mediated by the Barley stripe mosaic virus, Plant Mol. Biol. 81 (6) (2013) 595–608, https://doi.org/10.1007/s11103-013-0022-7.
[24] M. Knip, M.E. Constantin, H. Thordal-Christensen, Trans-kingdom cross-talk: small RNAs on the move, PLoS Genet. 10 (9) (2014) e1004602, https://doi.org/10.1371/journal.pgen.1004602.
[25] T. Mascia, F. Nigro, A. Abdallah, M. Ferrara, A. de Stradis, R. Faedda, P. Palukaitis, D. Gallitelli, Gene silencing and gene expression in phytopathogenic fungi using a plant virus vector, Proc. Natl. Acad. Sci. 111 (11) (2014) 4291–4296, https://doi.org/10.1073/pnas.1315668111.
[26] C. M Andrade, M.L. Tinoco, A.F. Rieth, F.C.O. Maia, F.J.L. Aragão, Host-induced gene silencing in the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*, Plant Pathol. 65 (4) (2016) 626–632, https://doi.org/10.1111/ppa.12447.
[27] M.C. Derbyshire, A.M. Gohari, R. Mehrabi, S. Kilaru, G. Steinberg, S. Ali, A. Baley, K. Hammond-Kosack, G.H.J. Kema, J. Rudd, Phosphopantetheinyl transferase (Ppt)-mediated biosynthesis of lysine, but not siderophores or DHN melanin, is required for virulence of *Zymoseptoria tritici* on wheat, Sci. Rep. 8 (1) (2018) 17069, https://doi.org/10.1038/s41598-018-35223-8.
[28] R.J. Howard, M.A. Ferrari, D.H. Roach, N. P Money, Penetration of hard substrates by a fungus employing enormous turgor pressures, Proc. Natl. Acad. Sci. 88 (24) (1991) 11281–11284, https://doi.org/10.1073/pnas.88.24.11281.
[29] R. Horbach, A. Graf, F. Weihmann, L. Antelo, S. Mathea, J.C. Liermann, T. Opatz, E. Thines, J. Aguirre, H.B. Deising, Sfp-type 4′-phosphopantetheinyl transferase is indispensable for fungal pathogenicity, Plant Cell 21 (10) (2009) 3379–3396, https://doi.org/10.1105/tpc.108.064188.
[30] H. Horiuchi, M. Fujiwara, S. Yamashita, A. Ohta, M. Takagi, Proliferation of intrahyphal hyphae caused by disruption of *csmA*, which encodes a class V chitin synthase with a myosin motor-like domain in *Aspergillus nidulans*, J. Bacteriol. 181 (12) (1999) 3721–3729, https://doi.org/10.1128/jb.181.12.3721-3729.1999.
[31] M. Martín-Urdíroz, M.I.G. Roncero, J.A. González-Reyes, C. Ruiz-Roldán, ChsVb, a class VII chitin synthase involved in septation, is critical for pathogenicity in *Fusarium oxysporum*, Eukaryot. Cell 7 (1) (2008) 112–121, https://doi.org/10.1128/ec.00347-07.

[32] A. Amnuaykanjanasin, L. Epstein, A class Vb chitin synthase in *Colletotrichum graminicola* is localized in the growing tips of multiple cell types, in nascent septa, and during septum conversion to an end wall after hyphal breakage, Protoplasma 227 (2) (2006) 155–164, https://doi.org/10.1007/s00709-005-0126-2.
[33] J.A. Crouch, B.B. Clarke, B.I. Hillman, What is the value of ITS sequence data in *Colletotrichum* systematics and species diagnosis? A case study using the falcate-spored graminicolous *Colletotrichum* group, Mycologia 101 (5) (2009) 648–656, https://doi.org/10.3852/08-231.
[34] N.L. Glass, G.C. Donaldson, Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes, Appl. Environ. Microbiol. 61 (4) (1995) 1323–1330, https://doi.org/10.1128/aem.61.4.1323-1330.1995.
[35] M.D. Templeton, E.H. Rikkerink, S.L. Solon, R.N. Crowhurst, Cloning and molecular characterization of the glyceraldehyde-3-phosphate dehydrogenase-encoding gene and cDNA from the plant pathogenic fungus *Glomerella cingulata*, Gene 122 (1) (1992) 225–230, https://doi.org/10.1016/0378-1119(92)90055-T.
[36] D.G. Higgins, J.D. Thompson, T.J. Gibson, [22] using CLUSTAL for multiple sequence alignments, Methods Enzymol. 266 (1996) 383–402, https://doi.org/10.1016/S0076-6879(96)66024-8. Academic Press.
[37] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (7) (2016) 1870–1874, https://doi.org/10.1093/molbev/msw054.
[38] S.Q. Le, O. Gascuel, An improved general amino acid replacement matrix, Mol. Biol. Evol. 25 (7) (2008) 1307–1320, https://doi.org/10.1093/molbev/msn067.
[39] S. Campo, K.B. Gilbert, J.C. Carrington, Small RNA-based antiviral defense in the phytopathogenic fungus *Colletotrichum higginsianum*, PLoS Pathog. 12 (6) (2016) e1005640, https://doi.org/10.1371/journal.ppat.1005640.
[40] W. Tang, S. Coughlan, E. Crane, M. Beatty, J. Duvick, The application of laser microdissection to in planta gene expression profiling of the maize anthracnose stalk rot fungus *Colletotrichum graminicola*, Mol. Plant Microbe Interact. 19 (11) (2006) 1240–1250, https://doi.org/10.1094/MPMI-19-1240.
[41] B.R. Frame, H. Shou, R.K. Chikwamba, Z. Zhang, C. Xiang, T.M. Fonger, S.E. K. Pegg, B. Li, D.S. Nettleton, D. Peo, K. Wang, *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system, Plant Physiol. 129 (1) (2002) 13–22, https://doi.org/10.1104/pp.000653.
[42] B. Frame, M. Main, R. Schick, K. Wang, Genetic transformation using maize immature zygotic embryos, in: Plant Embryo Culture: Methods and Protocols, Humana press, Totowa, NJ, 2010, pp. 327–341, https://doi.org/10.1007/978-1-61737-988-8_22.
[43] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method, Methods 25 (4) (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[44] N.F.J.D.A. Pinto, Controle químico da antracnose (*Colletotrichum graminicola*) do sorgo, International Journal of Maize & Sorghum 2 (3) (2003).
[45] R Core Team, R: a Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2016. http://www.R-project. org/.
[46] T. Olivoto, Lights, camera, pliman! an R package for plant image analysis, Methods Ecol. Evol. 13 (4) (2022) 789–798, https://doi.org/10.1111/2041-210X.13803.
[47] D. Lüdecke, M.S. Ben-Shachar, I. Patil, P. Waggoner, D. Makowski, Performance: an R package for assessment, comparison and testing of statistical models, J. Open Source Softw. 6 (60) (2021), https://doi.org/10.21105/joss.03139.
[48] L.J. Vaillancourt, R.M. Hanau, Genetic and morphological comparisons of *Glomerella* (*Colletotrichum*) isolates from maize and from sorghum, Exp. Mycol. 16 (3) (1992) 219–229, https://doi.org/10.1016/0147-5975(92)90030-U.
[49] N. Kalleda, A. Naorem, R.V. Manchikatla, Targeting fungal genes by diced siRNAs: a rapid tool to decipher gene function in *Aspergillus nidulans*, PLoS One 8 (10) (2013) e75443, https://doi.org/10.1371/journal.pone.0075443.
[50] R.P. da Silva, R. Puccia, M.L. Rodrigues, D.L. Oliveira, L.S. Joffe, G.V. César, L. Nimrichter, S. Goldenberg, L.R. Alves, Extracellular vesicle-mediated export of fungal RNA, Sci. Rep. 5 (1) (2015) 7763, https://doi.org/10.1038/srep07763.
[51] R. Asokan, G.S. Chandra, M. Manamohan, N.K.K. Kumar, T. Sita, Response of various target genes to diet-delivered dsRNA mediated RNA interference in the cotton bollworm, *Helicoverpa armigera*, J. Pest. Sci. 87 (1) (2014) 163–172, https://doi.org/10.1007/s10340-013-0541-7.
[52] S. Štefanić, J. Dvořák, M. Horn, S. Braschi, D. Sojka, D.S. Ruelas, B. Suzuki, K. Lim, S.D. Hopkins, J.H. McKerrow, C.R. Caffrey, RNA interference in *Schistosoma mansoni* schistosomula: selectivity, sensitivity and operation for larger-scale screening, PLoS Neglected Trop. Dis. 4 (10) (2010) e850, https://doi.org/10.1371/journal.pntd.0000850.
[53] M. Meyering-Vos, A. Müller, RNA interference suggests sulfakinins as satiety effectors in the cricket *Gryllus bimaculatus*, J. Insect Physiol. 53 (8) (2007) 840–848, https://doi.org/10.1016/j.jinsphys.2007.04.003.
[54] N.G. Bologna, O. Voinnet, The diversity, biogenesis, and activities of endogenous silencing small RNAs in arabidopsis, Annu. Rev. Plant Biol. 65 (1) (2014) 473–503, https://doi.org/10.1146/annurev-arplant-050213-035728.
[55] V.N. Kim, J. Han, M.C. Siomi, Biogenesis of small RNAs in animals, Nat. Rev. Mol. Cell Biol. 10 (2) (2009) 126–139, https://doi.org/10.1038/nrm2632.
[56] B. Czech, G.J. Hannon, Small RNA sorting: matchmaking for argonautes, Nat. Rev. Genet. 12 (1) (2011) 19–31, https://doi.org/10.1038/nrg2916.
[57] E. Allen, Z. Xie, A.M. Gustafson, J.C. Carrington, microRNA-directed phasing during trans-acting siRNA biogenesis in plants, Cell 121 (2) (2005) 207–221, https://doi.org/10.1016/j.cell.2005.04.004.
[58] C. Cogoni, G. Macino, Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase, Nature 399 (6732) (1999) 166–169, https://doi.org/10.1038/20215.
[59] S. Gazzani, T. Lawrenson, C. Woodward, D. Headon, R. Sablowski, A link between mRNA turnover and RNA interference in *Arabidopsis*, Science 306 (5698) (2004) 1046–1048, https://doi.org/10.1126/science.1101092.
[60] S. Astier-Manifacier, P. Cornuet, Purfication and molecular weight of an RNA-dependant RNA polymerase from *Brassicae oleracea* var. *Botrytis*, C. R. Hebd. Seances Acad. Sci. Ser. D Sci. Nat. 287 (11) (1978) 1043–1046.
[61] F. Boege, L.S. Heinz, RNA-dependent RNA polymerase from healthy tomato leaf tissue, FEBS (Fed. Eur. Biochem. Soc.) Lett. 121 (1) (1980) 91–96, https://doi.org/10.1016/0014-5793(80)81273-7.
[62] N. Romano, G. Macino, Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences, Mol. Microbiol. 6 (22) (1992) 3343–3353, https://doi.org/10.1111/j.1365-2958.1992.tb02202.x.
[63] P.K. Shiu, N.B. Raju, D. Zickler, R.L. Metzenberg, Meiotic silencing by unpaired DNA, Cell 107 (7) (2001) 905–916, https://doi.org/10.1016/S0092-8674(01)00609-2.
[64] R.L. Metzenberg, Meiotic silencing by unpaired DNA, NSF Award Number 0533093. Directorate for Biological Sciences 5 (533093) (2005) 33093, https://doi.org/10.1016/S0092-8674(01)00609-2.
[65] Y. Dang, Q. Yang, Z. Xue, Y. Liu, RNA interference in fungi: pathways, functions, and applications, Eukaryot. Cell 10 (9) (2011) 1148–1155, https://doi.org/10.1128/EC.05109-11.
[66] A. Dalakouras, M. Wassenegger, J.N. McMillan, V. Cardoza, I. Maegele, E. Dadami, M. Runne, G. Krczaal, M. Wassenegger, Induction of silencing in plants by high-pressure spraying of in vitro-synthesized small RNAs, Front. Plant Sci. 7 (2016) 1327, https://doi.org/10.3389/fpls.2016.01327.
[67] A.D. Coleman, S.T. Mugford, S.A. Hogenhout, Silencing of aphid genes by dsRNA feeding from plants, in: Management of Insect Pests to Agriculture: Lessons Learned from Deciphering their Genome, Transcriptome and Proteome, Springer International Publishing, Cham, 2016, pp. 245–251, https://doi.org/10.1007/978-3-319-24049-7_10.
[68] N. de Souza, Off-targets in RNAi screens, Nat. Methods 11 (5) (2014) 480, https://doi.org/10.1038/nmeth.2958, 480.
[69] S. Mosquera, M. Ginésy, I.T. Bocos-Asenjo, H. Amin, S. Diez-Hermano, J.J. Diez, J. Niño-Sánchez, Spray-induced gene silencing to control plant pathogenic fungi: a step-by-step guide, J. Integr. Plant Biol. 67 (3) (2025) 801–825, https://doi.org/10.1111/jipb.13848.
[70] L. Qiao, C. Lan, L. Capriotti, A. Ah-Fong, J. Nino Sanchez, R. Hamby, J. Heller, H. Zhao, N.L. Glass, H.S. Judelson, B. Mezzetti, D. Niu, H. Jin, Spray-induced gene silencing for disease control is dependent on the efficiency of pathogen RNA uptake, Plant Biotechnol. J. 19 (9) (2021) 1756–1768, https://doi.org/10.1111/pbi.13589.
[71] M.K. Gilbert, R. Majumdar, K. Rajasekaran, Z.Y. Chen, Q. Wei, C.M. Sickler, M. D. Lebar, J.W. Cary, B.R. Frame, K. Wang, RNA interference-based silencing of the alpha-amylase (amy1) gene in *Aspergillus flavus* decreases fungal growth and aflatoxin production in maize kernels, Planta 247 (6) (2018) 1465–1473, https://doi.org/10.1007/s00425-018-2875-0.
[72] D. Thakare, J. Zhang, R.A. Wing, P.J. Cotty, M.A. Schmidt, Aflatoxin-free transgenic maize using host-induced gene silencing, Sci. Adv. 3 (3) (2017) e1602382, https://doi.org/10.1126/sciadv.1602382.