

Decoding the RNAi machinery of *Ampelomyces* mycoparasites and their plant pathogenic and mycoparasitic relatives

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Abstract

The RNA interference (RNAi) machinery is present in some eukaryotes but absent in others, including certain model fungi. We performed *in silico* analyses and a proof-of-concept experiment using Spray-Induced Gene Silencing (SIGS) to demonstrate the presence and functionality of the RNAi machinery in *Ampelomyces* spp., common mycoparasites and biocontrol agents (BCAs) of powdery mildews – a group of obligate biotrophic fungi that includes important crop pathogens. The interactions between plants, powdery mildews, and *Ampelomyces* are classic examples of specific tritrophic relationships in the phyllosphere, and RNA–RNA crosstalk may play an important role in shaping these interactions. We identified core RNAi-related genes in the genomes of three phylogenetically diverse *Ampelomyces* strains and in the following relatives in the Dothideomycetes: *Parastagonospora nodorum* (*Pnod*), *Pyrenophora tritici-repentis* (*Ptri*) and *Zymoseptoria tritici* (*Ztri*), three economically important plant pathogens; and *Paraphaeosphaeria minitans* (*Pmin*), another mycoparasite and BCA used in crop protection. This is the first extensive study of RNAi machinery in *Pnod*, *Ptri* and *Pmin*. SIGS of a gene associated with mycoparasitism indicated that RNAi is functional in *Ampelomyces*. The results will enhance the understanding of the evolutionary relationships between *Ampelomyces* and their plant pathogenic and mycoparasitic relatives and may reveal new avenues for the application of *Ampelomyces* as BCAs.

Introduction

RNA interference (RNAi) is a mechanism in which specific short interfering RNA (siRNA) or microRNA (miRNA) molecules inhibit gene expression or translation by neutralizing the target messenger RNA (mRNA) molecules^{1–3}. Small-RNA (sRNA) facilitated RNAi is an evolutionarily conserved mechanism of self-protection which is triggered by a wide range of exogenic nucleic acids such as invasive viruses, transposons, transgenes, and plasmids^{4–6}. In fungi, the role of RNAi in genome defence against the insertion of repetitive transgenes during meiotic silencing of unpaired DNA and vegetative growth was first reported in *Neurospora crassa*⁷. Later, RNAi and its role in defence against retrotransposon activity has been revealed in diverse fungal species with distinct lifestyles⁸. In addition to this role, the fungal RNAi machinery produces a variety of sRNAs involved in the regulation of several biological processes through targeted gene silencing^{8–11}. The RNAi machinery is present in most eukaryotes but missing from others, including some fungi¹²: for example, the basidiomycete *Cryptococcus deuterogattii* does not possess it¹³ similar to several other fungi, including the model species *Ustilago maydis*¹⁴ and *Saccharomyces cerevisiae*¹⁵.

During the RNA silencing process, a series of key components, including the Argonaute proteins (AGOs), dicer-like proteins (DCLs), and RNA-dependent RNA polymerases (RDRs) produce 20 to 26 nucleotide (nt)-long sRNAs^{3,16–19}. Double-stranded RNA (dsRNA) precursors of sRNAs are generated by DCL proteins followed by their incorporation into the RNA Inducing Silencing Complex (RISC)^{16,17,20–22}. A large part of RNAi is performed by AGO proteins that become attached to sRNAs and intermingle with

homologous RNAs. This process suppresses the translation of mRNAs, which ultimately affect DNA methylation and endonuclease activity^{16,17,20,21,23}. RDRs use single-stranded RNAs as templates and synthesize dsRNA, which are then further processed by DCL proteins and initiate the second round of RNA silencing^{16,17,20,21}. The origin and formation process of sRNAs divide them into siRNAs and miRNAs^{24,25}.

sRNAs play a significant role in interactions between closely interacting organisms, cross-kingdom or within the same kingdoms, including mutualistic and parasitic interactions^{4,26–30}. sRNAs can be transferred cross-kingdom, e.g., during interactions between plants and their fungal pathogens, and induce gene silencing in both pathogens and their plant hosts that may open new frontiers in crop protection^{28,30–32}. This phenomenon is known as cross-kingdom RNA interference (CKRI)^{4,5,33–35} and has prompted Spray Induced Gene Silencing (SIGS) experiments as a new approach for the management of crop diseases. SIGS is based on RNAi and applies dsRNA molecules that are able to silence specific genes in the target plant pathogens^{32,36}. The dsRNA used in SIGS is synthesized *in vitro* and sprayed on plants infected with fungal or other microbial pathogens. Fungi and other plant pathogens can absorb dsRNA directly from the sprayed materials; or, dsRNA is first absorbed by the infected plant tissues that process them and produce sRNAs that are then transferred from the plants to the pathogen^{36,37}. SIGS has some limitations, including the stability of the naked siRNA³⁸. The stability of the naked siRNA can be increased by using bioclay or nanoparticles³⁹; or encapsulating siRNA in artificial extracellular vesicles³⁴.

The role of sRNAs has also been studied in mycoparasitic interactions that are specific antagonistic relationships between fungi and oomycetes known as mycoparasites, and their fungal hosts known as mycohosts^{40–44}. The mycoparasitic nutrition mode is relatively little understood⁴⁰ although some mycoparasites have already been commercialized as biocontrol agents (BCAs) of crop pathogens^{45,46} and others cause economic damage in mushroom production^{47,48}. RNAi has been studied in detail in interactions between *Clonostachys rosea*, a model mycoparasite, and two of its mycohosts, *Fusarium graminearum* and *Botrytis cinerea*^{10,49–51}; and also, between *Trichoderma atroviride* and its mycohosts *Rhizoctonia solani* and *Alternaria alternata*⁵². RNAi machinery components AGO3 and DCL2 of *T. atroviride* have been described playing a role in host recognition and mycoparasitism by gene regulation, defence, and degradation of the mycohosts' hyphal walls⁵². Piombo et al.⁵¹ provided a comparative analysis of the core proteins in the RNAi pathway in fungal and oomycete species parasitizing insects and fungi, including the mycoparasites *C. rosea*, *C. rhizophaga*, *C. solani*, *T. asperellum*, and *T. virens*.

Here, we present the first study of the RNAi machinery in *Ampelomyces* spp. mycoparasites, common natural enemies of all species of powdery mildews (Erysiphaceae)^{53–56}. Powdery mildews are ubiquitous obligate biotrophic pathogens of over 10,000 dicot and monocot species, including important crops such as wheat, barley, grapevine, and fruit and vegetable species^{57,58}. Strains of *Ampelomyces* spp. have long been studied as potential BCAs of powdery mildews, and some strains have already been

commercialized to manage powdery mildew infections of grapes and vegetables^{46,55,59}. Hyphae of *Ampelomyces* are uniquely capable of penetrating powdery mildew hyphae and conidia on the host plant surfaces^{53,55,60}. Following penetration, *Ampelomyces* hyphae continue their growth and development inside the hyphae of powdery mildew colonies, consuming their cytoplasm and develop their own asexual fruiting bodies known as pycnidia mostly in the powdery mildew conidiophores, and young, immature fruiting bodies, i.e., chasmothecia^{46,53,55,59–63}. The taxonomy of the genus *Ampelomyces* is still unresolved^{46,64–66}. Strains isolated from diverse powdery mildew species in different parts of the world are genetically diverse^{54,64–66}; phylogenetic analyses have indicated that they belong to up to nine molecular operational taxonomic units^{67,68}.

Ampelomyces mycoparasites are phylogenetically related to several well-known crop pathogens within the Dothideomycetes, including *Parastagonospora nodorum* (*Pnod*), *Pyrenophora tritici-repentis* (*Ptri*), and *Zymoseptoria tritici* (*Ztri*); and also to another common mycoparasite, *Paraphaeosphaeria minitans* (syn. *Coniothyrium minitans*) (*Pmin*)^{40,67,69}. Interestingly, the RNAi machinery remains insufficiently studied in all these fungi except *Ztri*^{70,71}.

Genomic resources are available for a few *Ampelomyces* strains^{67–69}. High quality genome assemblies and annotations are available for some of the closely related species, *Pnod*, *Ptri*, *Ztri*, and *Pmin*^{69,72–75}. These resources provide opportunities for identification of the RNAi machinery in *Ampelomyces* and its close plant pathogenic and mycoparasitic relatives *in silico*. If identified, key components of RNAi could be knocked out in *Ampelomyces* to reveal their potential role in mycoparasitism. A molecular toolbox for genetic transformations of *Ampelomyces* using *Agrobacterium*-mediated transformation^{60,63} and also with CRISPR-Cas9 (Goldspink, Kiss & Idnurm, unpublished) have already been developed.

In this work, we hypothesize that *Ampelomyces* mycoparasites produce sRNAs that target their mycohosts, the obligate biotrophic powdery mildews; and if this is the case, such powdery mildew-specific siRNAs could potentially be developed for biocontrol purposes. Piombo et al.⁵¹ have already indicated that sRNAs deployed by fungal BCAs to suppress crop pathogens and insect pests could be explored for crop disease and insect pest management. Also, a comparative study of the RNAi machinery in closely related crop pathogens and mycoparasites may contribute to a better understanding of their evolution that has led to distinct nutrition modes. Some of these fungi, i.e., *Pnod*, *Ptri*, and *Ztri*, attack and feed on plants; while *Ampelomyces* and *Pmin* attack and feed on fungi⁴⁰. Each of these fungi is specialised to infect and feed on a restricted number of plant or fungal hosts only, which make them an interesting target for evolutionary studies.

The specific objectives of this study were to (i) identify the RNAi machinery in three *Ampelomyces* strains with high quality genome assemblies, and representing different Molecular Operational Taxonomic Units (MOTUs), focusing on AGOs, DCLs and RDRs; (ii) identify AGOs, DCLs and RDRs in closely related plant pathogenic species, *Pnod*, *Ptri*, and *Ztri*, and a closely related mycoparasite, *Pmin* and compare with

Ampelomyces; and (ii) investigate whether the RNAi machinery in *Ampelomyces* is functional by using SIGS.

Results

Components of RNAi machinery are present in closely related mycoparasites and plant pathogens included in this study

To identify the RNAi machinery in *Ampelomyces* sp. strain HMLAC 05119, protein sequences of RNAi machinery components of *Blumeria graminis* f. sp. *tritici* (*Bgt*), a model obligate biotrophic plant pathogen causing wheat powdery mildew⁷⁶⁻⁷⁸, and *Arabidopsis thaliana*, a model plant species⁷⁹, were used as queries in BLAST searches against the genome. Accession numbers are provided in Supplementary Table S1. Sequences of identified AGOs, DCLs and RDRs in *Ampelomyces* strain HMLAC 05119 were used to identify AGOs, DCLs and RDRs in the other two *Ampelomyces* strains (BRIP 72097 and BRIP 72107), and the plant pathogenic relatives *Pnod*, *Ptri*, and *Ztri*, and also in *Pmin*, a closely related mycoparasite.

Based on similarity and query coverage, all RNAi machinery components revealed in *Bgt*⁷⁸ were identified in *Ampelomyces* strain HMLAC 05119. Some of the *Bgt* components were annotated correctly, while others were annotated based on the names of active domains in the respective proteins. Four components were labelled as hypothetical proteins⁷⁸ (Table 1). Sequence-based BLAST searches identified two Argonautes (AGO1 and AGO2), two Dicers (DCL1 and DCL2), three RDRs (RdRP1, RdRP2 and RdRP3), three Quelling Defective proteins 3 (QDE3)-like helicases (including one hypothetical protein and two P-loop-containing nucleoside triphosphate hydrolases), replication factor A (RPA1), a DNA methylase (hypothetical protein), an RNA-Induced Silencing Complex nuclease (RISC) (hypothetical protein), a QDE-2-interacting protein (QIP) (hypothetical protein) RNase III (ribonuclease III-like domain-containing protein), and an RNase inhibitor (Argonaute siRNA chaperone complex subunit Arb1-domain-containing protein) in *Ampelomyces* strain HMLAC 05119 (Table 1). Sequences of AGOs, DCLs and RDRs were analyzed using protein signature databases to identify their domain architectures. The results were presented along with the ITS phylogeny of the seven strains included in this study (Figs. 1A, 2A and 3A).

We were able to trace two homologs of AGO genes in all three *Ampelomyces* strains (HMLAC 05119, BRIP 72097 and BRIP 72107) and also in *Pnod* and *Pmin* genomes, and three AGOs in *Ptri* and *Ztri* (Fig. 1A). Most AGO-coding genes across all these species possessed five conserved domains: Argonaute N-terminal (IPR032474), Argonaute linker 1 (IPR014811), PAZ (IPR003100), AGO linker 2 (IPR032472), and Piwi (IPR003165). An exception was *Ztri*_AGO3, which lacked the Argonaute N-terminal domain (IPR032474). Argonaute Mid domain (IPR032473) was only present in the *Ampelomyces* AGO1 but not in close relatives (Fig. 1A). A cladogram of AGO sequences revealed that AGO1 and AGO2 genes clustered into distinct clades, while AGO3 grouped within the AGO2 clade (Fig. 1B).

Two DCL homologs were identified in the three *Ampelomyces* strains, and also in *Pmin*, *Ptri*, and *Pnod*, whereas only one was found in *Ztri* (Fig. 2A). All DCLs contained four common domains namely the DEAD/DEAH box helicase domain (IPR014001, IPR011545, IPR033517, IPR044113, IPR006935), the helicase C-terminal domain (IPR001650), the Dicer dimerization domain (IPR005034) and the ribonuclease III domain (IPR000999). The dsRNA binding domain was present in one copy of DCLs of all genomes except *Ptri*; and absent in the other DCL homolog. DCL copies which did not have dsRNA binding domain possessed Piwi, Argonaute and Zwiller/Pinhead (PAZ) domain (IPR003100) in all genomes except *Ztri*, in which all six domains were present (Fig. 2A). A cladogram of all DCL sequences indicated that DCL1 and DCL2 belong to distinct, monophyletic clades (Fig. 2B). DCL1 and DCL2 sequences of *Ampelomyces* grouped together within both DCL clades and were closely related to *Pnod*. The DCLs of *Ptri*, *Pmin* and *Ztri* were more distantly related to *Ampelomyces* and *Pnod* in both clades. The grouping of the seven strains based on DCLs (Fig. 2B) mirrored the phylogenetic relationships revealed by the AGO analysis (Fig. 1B).

Three copies of RDRs were identified in all seven genomes except *Ptri*, which had only two. All RDR genes contained the RNA-dependent RNA polymerase domain (Fig. 3A). A cladogram of RDR sequences resolved three distinct clades, with homologous RDRs from different strains clustering together to indicate the monophyletic origin of each clade (Fig. 3B). The grouping of the strains mirrored that of the DCLs, except for RDR2, where *Ztri* RDR2 was more closely related to *Ampelomyces* RDR2 than to those of *Pnod* and *Ptri*.

We also studied the presence of plant RNAi components in *Ampelomyces*. Out of 23 *Arabidopsis* RNAi machinery components²⁵, the following 12 were retrieved from *Ampelomyces* HMLAC 051119, under different names: Serate (SE) (SMAD/FHA domain-containing protein), Negative on tata less 2 (NOT2) (fungal-specific transcription factor domain-containing protein), Cell Division Cycle 5 (CDC5) (pre-mRNA splicing factor component-domain-containing protein), C-terminal domain phosphatase-like 4 (CPL4) (serine/threonine-protein phosphatase PP2A catalytic subunit), Cap-Binding protein (CBP), Stabilized1 (STA1) (PRP1 splicing factor, N terminal domain containing protein), Pleiotropic Regulatory Locus 1 (PRL) (WD40-repeat-containing domain protein), Modifier of SNC1, 2 (MOS2) (DEXH-box splicing factor binding site-domain-containing protein), Psr1-interacting protein 1 (PINP1) (P-loop containing nucleoside triphosphate hydrolase protein), RNA Lariat Debranching Enzyme 1 (DRB1) (lariat debranching enzyme), Heat Shock Protein 90 (HSP90), and Chromatin Remodeling Factor 1 (CLASSY1) (P-loop containing nucleoside triphosphate hydrolase protein) (Table 2).

The Hyponastic Leaves 1 (HYL1), Hua Enhancer 1 (HEN1), Suppressor of Gene Silencing 3 (SGS3), Dicer RNA binding factor 4 (DRB4), regulator of CBF gene expression 3 (RCF3), MOS4-Associated Complex (MAC), THO/TREX Complex Subunit 7 (Tho), SICKLE (SKL), Transportin 1 (TRN1), Enhanced miRNA activity1 (EMA1) and Defective in RNA-Directed DNA Methylation 1 (DRD1) components of *Arabidopsis* RNAi machinery were not found in *Ampelomyces*. Most of the *Arabidopsis* RNAi components that were not found in *Ampelomyces* belonged to the miRNA pathway. This may indicate that the miRNA pathway in *Ampelomyces* is somewhat different from the plant miRNA pathway.

In total, 49 DCLs, RDRs and AGO proteins were identified in *Ampelomyces* and its four close relatives. The annotation of all these 49 genes was manually verified, and we found that 17 out of 49 genes needed re-annotations. Our re-annotated DNA and protein sequences were included in Supplementary Files S1 and S2. . The re-annotated genes were RDR3 in *Pnod*, AGO3 and RDR3 in *Pmin*, AGO3 and RDR2 in *Ptri*, AGO1, AGO3, RDR1, RDR2 and RDR3 in *Ztri*, DCL2, AGO1, AGO2, RDR2, and RDR3 in *Ampelomyces* strain BRIP 72097 and DCL2 in *Ampelomyces* strain BRIP 72107 (Supplementary Tables S2 and S3, Supplementary Files S1 and S2). No genes needed to be reannotated in *Ampelomyces* strain HMLAC 05119.

The RNAi machinery is functional in *Ampelomyces* strain BRIP 72097

To assess whether the RNAi machinery in *Ampelomyces* is functionally active, a SIGS proof-of-concept experiment was conducted with strain BRIP 72097. An α -1,3-glucanase gene (*exgA*) was selected as the SIGS target because *exgA* is the first – and so far the only – gene known to be associated with mycoparasitic activity in *Ampelomyces*⁸⁰. The 2746 bp long *exgA* mRNA sequence available in NCBI GenBank from the original study⁸⁰ (AF029354) indicated that the coding (CDS) region is between 273 bp and 2606 bp. A 309 bp fragment of a less variable region was selected to synthesize a 355 bp long *exgA*-targeting dsRNA for SIGS (Figs. 4 and 5, Supplementary Files S3 and S4). This dsRNA was sprayed onto growing colonies of BRIP 72097 in culture to evaluate whether this *Ampelomyces* strain can produce an RNAi-mediated gene silencing response.

Total RNA was extracted from dsRNA-treated and control *Ampelomyces* colonies and cDNA was produced by reverse transcription polymerase chain reaction (RT-PCR). In dsRNA-treated colonies, quantitative real-time PCR (qPCR) of the cDNA samples indicated a significant reduction of *exgA*-mRNA at three- and six-days post treatment (dpt) ($P < 0.01$ and $P < 0.05$, respectively). Nine and twelve dpt, the *exgA*-mRNA levels were not significantly different in the dsRNA-treated versus control colonies (Fig. 6).

These results indicate that the *Ampelomyces* strain absorbed the externally applied dsRNA molecules and its RNAi machinery likely converted them into siRNA fragments that suppressed the expression of *exgA* for up to six days. The hypothetical steps of this process are shown in Fig. 6. We did not detect any differences in the colour, development, growth and other morphological patterns of the dsRNA-treated colonies in plates and their hyphal and conidial morphology observed with light microscopy during the experiment, up to 12 dpt; and neither 15, 20 and 25 dpt.

Discussion

sRNAs derived from fungal biocontrol agents may offer new strategies for managing crop pathogenic fungi

RNAi may open new avenues for environmentally friendly approaches in various areas of crop protection including management of plant viruses and insect pests⁸¹⁻⁸³. sRNAs identified in plant–pathogen interactions and other cross–kingdom relationships have already been explored for their potential

application in crop protection^{28,29,31-36}. The role of sRNAs in fungal–fungal interactions was recently described as a “new paradigm”, and it remains a largely unexplored field that may also offer new opportunities for controlling crop pathogenic fungi through RNAi delivered by their fungal BCAs⁵¹.

Within this field, the first step is to decipher whether biocontrol fungi possess the RNAi machinery. Core proteins of RNAi were identified in some key fungal biocontrol agents including *Clonostachys* and *Trichoderma* spp.⁴⁹⁻⁵². This is important because some fungi, including model species, lack the RNAi machinery¹²⁻¹⁵. In this work, we report the AGOs, DCLs and RDRs in three phylogenetically diverse strains of *Ampelomyces* mycoparasites that have long been used as BCAs of powdery mildews^{46,53-56}, a large group of obligate biotrophic plant pathogens that are notoriously difficult to control in many crops⁵⁸. We hypothesize that *Ampelomyces* mycoparasites deploy sRNAs during their attack on the obligate biotrophic powdery mildews. Once identified, these anti-powdery mildew sRNAs may be synthesised and tested for their use in crop disease management as molecules derived from a biocontrol fungus.

Characteristics of the RNAi machinery in mycoparasites and plant pathogens included in this study

Fungi can generate sRNAs through DCL-dependent and DCL-independent pathways⁸⁴. The generated sRNAs are then loaded into AGOs which guide them to base pairing with target RNA for sequence-specific gene silencing⁸⁵. Gene silencing could be the result of translation inhibition, RNA degradation, or chromatin modification; during these processes diverse dsRNAs are processed by different DCLs to produce various sRNAs that are then loaded on different AGOs. Some sRNAs can be generated directly by RDRs⁸⁶. The *in silico* evidence of RDRs in all the seven strains included in this work indicates that they can produce AGO-independent sRNAs.

In addition to *Ampelomyces*, this is also the first comprehensive study of the RNAi machinery in *Pnod*, *Ptri* and *Pmin*. In *Ztri*, AGOs, DCLs, and also a DCL-independent pathway of sRNA biogenesis were identified⁷⁰ and it was shown that AGO1 affects the virulence of *Ztri*⁷¹. Our *in silico* study followed a previous work that identified active domains of AGOs, DCLs and RDRs in mycoparasites and entomopathogenic fungi⁵¹. All AGOs identified in this study and the AGOs of mycoparasites included in the previous work⁵¹ contained five domains except *Ztri*_AGO3 where the Argonaute N-terminal domain (IPR032474) was not identified. The Argonaute Mid domain (IPR032473) as an extra domain was present in one copy of AGOs in *Ampelomyces*. It was also identified in one copy of AGOs of two entomopathogens (*Metarhizium anisopliae* and *Metarhizium acridum*) and in both copies of AGOs of the oomycetes *Pythium oligandrum* and *Pythium periplocum*⁵¹. Interestingly, while the Argonaute Mid domain was present in all three strains of *Ampelomyces* AGO1 proteins, it was absent from the AGO proteins of *Clonostachys* and *Trichoderma* species⁵¹. These findings suggest differences in AGO domain architecture among mycoparasitic fungi, which may reflect functional diversification of their RNAi machinery.

The PAZ domain was found in Dicer proteins and Piwi family proteins that contribute to post-transcriptional gene silencing⁸⁷. PAZ may contribute to the precise and productive integration of miRNAs and siRNAs into the RNAi pathway^{87,88}. We identified the PAZ domain (IPR003100) in one copy of DCLs of *Pnod* and *Ztri*, and also in *Ampelomyces* and *Pmin*. This domain was not revealed earlier in other mycoparasites⁵¹.

The fungal RNAi machinery does not only consist of AGOs, DCLs and RDRs – it also includes QDE, RPA, QIP, DNA methylase, RISC nuclease, RNaseIII and RNase III inhibitor. All these fungal RNAi components had been identified e.g., in different powdery mildews⁷⁸ and we found them in HMLA 05119. When we searched for the 23 RNAi components known in *Arabidopsis*²⁵, only 12 were found in HMLAC 05119. The plant RNAi components that were not found in *Ampelomyces* were mostly from the miRNA biosynthesis pathways, i.e., HYL1, HEN1, RCF3, Mac, Tho, Sickie, TRN1, and EMA1. Plant and fungal miRNA production pathways may be different; in fungi, diverse pathways generate microRNA-like RNAs (milRNAs) and Dicer-independent siRNAs^{84,89}. In *Verticillium nonalfalfae*, precursors of miRNAs were transcribed by RNA polymerase III – found in *Ampelomyces* – then processed by DCLs to synthesise milRNAs⁸⁹. Some pathways that generate milRNAs require both QDE-2 and QIP for activation, others rely solely on QDE-2, and a few do not require either QDE-2 or QIP⁸⁴. As QDE-2 and QIP were found in *Ampelomyces*, we hypothesise that *Ampelomyces* can generate diverse types of siRNAs.

SIGS as a tool for proof-of-concept experiments

Numerous experiments had demonstrated that exogenous application of specific synthetic dsRNAs through SIGS silenced the target genes in the treated fungal mycelia^{32,36,37,39,90,91}. In our small-scale proof-of-concept experiment, the target gene, *exgA*, was downregulated for at least six dpt, which indicated that the RNAi machinery is functional in *Ampelomyces*. The impact of the SIGS treatment was transient as the *exgA*-mRNA levels at nine and twelve dpt were similar to the untreated control. Other studies have also found that the impact of “naked” dsRNA used in SIGS does not last long^{28,35,90-93}.

SIGS offers a relatively rapid and non-transgenic strategy for proof-of-concept experiments aimed at testing the functional roles of selected genes or, as in the present study, the functionality of biosynthetic pathways – in our case, the production of siRNAs. In contrast, the classical approach requires generating stable transgenic lines, which is often time-consuming, technically challenging, and limited to species that are amenable to transformation. In mycoparasites, gene knockout was used to demonstrate the role of RNAi in regulating mycoparasitic interactions between *C. rosea* and two of its mycohosts, *B. cinerea* and *F. graminearum*¹⁰. In *Ampelomyces*, gene knockout was applied to study the impact of a nitrate-reductase gene in mycoparasitism⁶³.

This study has the potential to stimulate further research on RNAi in mycoparasites and fungal BCAs. Such work may enhance our understanding of the evolutionary relationships between closely related mycoparasites and plant pathogens, and it may also lead to practical applications of RNAi in fungal

BCAs – for example, by harnessing the siRNAs these fungi use to suppress their targets and applying these molecules in the management of crop diseases and other pest-management contexts. In particular, this research may uncover new opportunities to exploit the biocontrol potential of *Ampelomyces* for the suppression of powdery mildew infections across a wide range of crops.

Materials and Methods

Identification of the RNAi machinery and domain prediction

For the RNAi machinery identification, protein sequences of *Blumeria graminis* f. sp. *tritici* (*Bgt*) and *Arabidopsis* were retrieved from GenBank database (accessions in Supplementary Table S1). These sequences were used as queries to search through genomic/proteomic databases of *Ampelomyces* strains BRIP 72097, BRIP 72107, and HMLAC 05119, and their close relatives, *Pnod*, *Ptri*, *Ztri*, and *Pmin*. Based on similarity and query coverages, the sequences of the hits were retrieved and used to search for all active domains using Interpro⁹⁴. Sequences of AGO, DCL and RDR proteins were analysed for domain content, and only those sequences containing at least three domains matching those reported in a recent study were retained⁵¹.

To verify the annotation of the retrieved protein coding RNA sequences (CDS) and protein sequences, genomic and transcriptomic data of *Ampelomyces* strains BRIP 72097, BRIP 72107, and HMLAC05119, and their Dothideomycete relatives, *Pnod*, *Ptri*, *Ztri*, and *Pmin*, were downloaded from NCBI. Supplementary Tables S2 and S3 list the accession numbers of genome assemblies, annotation files, CDS, proteins and RNA sequencing data included in our analyses. RNA data, genome assemblies and annotation files were uploaded to Galaxy server⁹⁵. RNA software STAR (Galaxy Version 2.7.11a+galaxy1) was used to map the RNA reads to the genome. BAM files were downloaded from Galaxy server and viewed using Integrative Genomics Viewer (IGV version 2.19.1)⁹⁶. FASTA files containing all the proteins and transcripts were used in Geneious tool (<http://www.geneious.com>) to make local data-bases for local BLAST. After creating the databases all the retrieved proteins and mRNAs were searched through the local databases and then checked the location of the coding genes in respective genomes.

RNA sequencing data mapped onto the respective genomes were used to confirm start codon, stop codon, exons and introns of all the CDS. Mapping data also provided the splice junctions information about the start and end of the exons and introns. Splice junction information was necessary to find out true exons, introns and their correct length. Some of the genes were re-annotated manually using IGV and the RNA sequencing data, as they were found to include in-frame stop codons or were not starting or ending appropriately. The re-annotation details can be found on Supplementary Table S4.

Phylogenetic analyses

Phylogenetic analyses were conducted using the following datasets. A single-locus phylogeny was inferred from sequences of the nrDNA ITS region of BRIP 72097, BRIP 72107, HMLAC05119, *Pnod*, *Ptri*, *Ztri*, and *Pmin* strains. Sequences were collected by using the *Ztri* ITS as a query to retrieve the rest of the

ITS sequences from the aforementioned genomes, including from the outgroup *Neurospora crassa* OR74A genome (GenBank ID: GCA_000182925.2). To achieve that, we used BLAST searches to identify the rest of the sequences from the respective genomes included in this study (Supplementary File S5). The sequences were first aligned using ClustalW (v1.83)^{97,98}, then trimmed and re-aligned. ClustalX (v2.1) was used to visualize the alignment and convert the alignment “.aln” file to a nexus file. Phylogenies were inferred using MrBayes (v3.2.7a x86_64)⁹⁹ with the parameters “lset nst=6 rates=invgamma” and keeping the average standard deviation of split frequencies below 0.025 for all trees.

Then, each RNAi machinery protein group in focus of this study, i.e., the AGOs, DCLs, and RDRs, was analysed based on the amino acid sequences of the already annotated and some re-annotated genes (Supplementary Tables S3 and S4). *N. crassa* protein DCL2 (XP_963538.3) was used as an outgroup in these analyses. We aligned the sequences using ClustalW, and then ClustalX, and then inferred the phylogenies using MrBayes as described for the ITS phylogeny. We visualised all trees using Figtree (v1.4.4) (<http://tree.bio.ed.ac.uk/software/figtree/>).

Fungal material preparation for SIGS

The *Ampelomyces* strain BRIP 72097 was selected for the SIGS work because it grew and sporulated well in culture as described previously⁶⁸. The strain was subcultured on Czapek-Dox medium supplemented with 2% malt (MCzA)⁵⁹; and kept in dark at 22°C. 6-cm-diameter plates with 15 days old *Ampelomyces* colonies were used in SIGS experiments.

Construct design and synthesis

We choose *exgA* to silence by using SIGS because *exgA* is the first gene associated with mycoparasitic activity in *Ampelomyces*⁸⁰. For SIGS the 2,746 bp long CDS sequence of the *exgA* mRNA (AF029354.1) was downloaded from NCBI. Because *exgA* protein was only 777 amino acid long, the NCBI entry was shortened by removing 272 bp at 5'-end and 140 bp at 3'-end of the original sequence available in Genbank. We identified *exgA* genes from BRIP 72097 and BRIP 72107 genomes⁶⁸ by using the AF029354.1 sequence. We aligned sequences by using Clustal Omega multiple sequence alignment tool online (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>) (Supplementary File S4). . We selected 309 bp from the less variable region (1719-2028) and added cutting sites and T7 promotor on both sides of the selected sequence (Supplementary File S3). The T7 promoter is recognized by T7 RNA polymerase and starts transcription. The designed construct was synthesized by Macrogen (South Korea) and delivered in a pMG-AMP plasmid. T7 RNA polymerase transcribed the single stranded RNA (ssRNA) which then hybridized into dsRNA (Figure 4). To generate the dsRNA designed for SIGS, MEGAscript® (ThermoFisher Scientific) RNAi kit was used to transcribe the dsRNA designed to target *Ampelomyces exgA* mRNA. 20 µL reaction was prepared by adding 2 µL of each component, 10 × T7 Reaction Buffer, nucleoside triphosphates (ATP, CTP, GTP, and UTP), T7 Enzyme Mix, 6 µL of nuclease-free water and dsRNA construct DNA (1–2 µg) to target *Ampelomyces exgA* mRNA. Reaction was gently mixed by pipetting,

then centrifuged at 1000 RPM for 30 seconds to gather the reaction mix at the bottom of the tube. Transcripts were bigger than 250bp so according to kit protocol elongated incubation (up to ~16 h) was given at 37°C. After incubation time, the concentration of the synthesized dsRNA was determined using a DS11 spectrophotometer (DeNovix, Wilmington, USA) and was diluted to 100 ng/μL. This concentration was applied based on preliminary experiments (data not shown).

Fifteen-days-old BRIP 72097 colonies grown on MCzA in 6 cm plates were sprayed with dsRNA, 100 ng/μL, using sterile 2 mL plastic spray bottles. Each plate was sprayed with 0.5 mL dsRNA and left to dry in a biosafety cabinet; then covered again with their original lids and kept in an incubator at 22°C in dark until required for sampling as described below. A total of 12 plates were treated in this way with dsRNA. In addition, 12 plates served as controls and were sprayed with nuclease free water. Two preliminary tests, one on plates and another in liquid culture were done before the final experiment was carried out twice. As the dsRNA uptake may require up to 12 hours¹⁰⁰ (Nerva et al., 2020), then the RNAi machinery processes it into short siRNAs which are loaded onto RISC complex and then target the mRNA (Smaghe, 2025). Spraying long dsRNA may take 72 hours to produce enough siRNAs which neutralize the mRNA on a detectable level (Song et al., 2018).

RNA extraction, cDNA preparation and qPCR

Ampelomyces cultures treated in the SIGS experiments were sampled three, six, nine and 12 dpt. The first sampling time was selected because the dsRNA uptake may require up to 12 hours¹⁰⁰; and in case of long dsRNA molecules, it may take 72 hours to produce enough siRNAs that neutralize the target mRNA at a detectable level¹⁰¹. Twenty-four samples were collected in total, six samples on each time point, i.e., one sample from each plate at one time point. Total RNA was extracted from 100 mg fresh mycelium scraped off each colony to avoid agar contamination of the samples and using the RNeasy Plant Mini Total RNA Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The RNA quality and quantity was analysed using DeNovix DS-11 (Wilmington, USA). From three replicates one sample with high quality RNA was used for qPCR. Two μg of the extracted RNA was used to synthesise cDNA using SensiFAST cDNA Synthesis Kit (Bioline Meridian Bioscience) according to the manufacturer's protocol. Synthesised cDNA was diluted to get 50 ng/μL and used for qPCR. Primers for qPCR were designed using PrimerQuest™ and ordered from MacroGen Inc. (Forward primer exgF: 5'-TGAAGTGGAACTGGCTGTG-3'; Reverse primer exgR: 5'-GGGTGTTGGTGAATTTGCTG-3'). For internal control target to normalize the qPCR, we used the actin gene (Forward primer actF: 5'-TCTTGAAAGCGGTGGTATCC-3'; Reverse primer actR: 5'-ATGACGATGTTGCCGTAGAG-3'). iTaq™ Universal SYBR® Green Supermix f (BioRad Hercules, California, USA) and BioRad CFX 96 qPCR machine were used for qPCR.

The expression levels of target genes were normalized against the actin gene to account for sample-to-sample variation. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method¹⁰². Each reaction was performed in technical triplicates, and the experiment was repeated twice. Data were analysed and

statistical significance was determined using Student's t-test. A result was considered statistically significant when $p < 0.05$.

Declarations

Data Availability

Benefits from this research accrue from the sharing of our data and results on public databases (SRA, NCBI) as described above. The datasets created and used in this study are provided in Supplementary Tables S1-S4 and Supplementary Files S1-S5.

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Author Contributions

L.K., A.G.S. and A.A. developed the study conception and design. A.A. and K.K. carried out the laboratory work; A.A., A.G.S., S.B., L.G., A.I., N.V. and L.K. performed the data analyses and data interpretation. L.K. and A.I. acquired the funding for the project. A.A. wrote the first version of the manuscript and L.K., A.G.S., L. G., A.I. and N.V. revised the manuscript and discussed the results. All authors read and approved the final manuscript.

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Competing Interests / Conflict of Interest Statement

The authors declare no competing interests.

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Tables

Table 1. Proteins of the RNAi machinery of *Blumeria graminis* f. sp. *tritici* (Bgt) as revealed by Kusch et al.⁷⁸ and used as references to identify the RNAi components in *Ampelomyces* strain HMLAC 05119.

RNAi proteins of <i>Bgt</i>	RNAi proteins of <i>Ampelomyces</i> HMLAC 05119
Dicer like protein 1(DCL1)	DCL1
Dicer like protein 2(DCL2)	DCL2
Ribonuclease (RNase III)	Ribonuclease-III-like-domain-containing protein
Argonaute protein 1 (AGO1)	Ribonuclease H-like domain-containing protein
Argonaute protein 2 (AGO2)	Piwi domain-containing protein
Argonaute subunit Arb1	Argonaute siRNA chaperone complex subunit Arb1-domain-containing protein
RNA-dep-RNA polymerase 1 (RdRP1)	RNA dependent RNA polymerase-domain-containing protein
RNA-dep-RNA polymerase 2 (RdRP2)	RNA dependent RNA polymerase-domain-containing protein
RNA-dep-RNA polymerase 3 (RdRP3)	RNA dependent RNA polymerase-domain-containing protein
RNA Inducing Silencing Complex (RISC) nuclease	Hypothetical protein BDU57DRAFT_536332
QDE3-like helicase 1 (QDE3-1)	Hypothetical protein BDU57DRAFT_464888
QDE3-like helicase 2 (QDE3-2)	P-loop containing nucleoside triphosphate hydrolase protein
QDE3-like helicase 3 (QDE3-3)	P-loop containing nucleoside triphosphate hydrolase protein
Replication factor A1 (RPA1)	Replication protein-like protein A
DNA methylase (DNMT)	Hypothetical protein BDU57DRAFT_547894
QDE-2-interacting protein (QIP)	Hypothetical protein BDU57DRAFT_494404

Table 2. RNAi machinery components from *Arabidopsis* used to identify homologous elements in *Ampelomyces* strain HMLAC 05119.

RNAi proteins of <i>Arabidopsis</i>	RNAi proteins of <i>Ampelomyces</i> HMLAC 05119
Serate (SE)	SMAD/FHA domain-containing protein
Negative on tata less 2 (NOT2)	fungal-specific transcription factor domain-containing protein
Cell Division Cycle 5 (CDC5)	pre-mRNA splicing factor component-domain-containing protein
C-terminal domain phosphatase-like 4 (CPL4)	serine/threonine-protein phosphatase PP2A catalytic subunit
Cap-Binding protein (CBP)	CBP
Stabilized1 (STA1)	PRP1 splicing factor, N terminal domain containing protein
Pleiotropic Regulatory Locus 1 (PRL)	WD40-repeat-containing domain protein
Modifier of SNC1, 2 (MOS2)	DExH-box splicing factor binding site-domain-containing protein
Psr1-interacting protein 1 (PINP1)	P-loop containing nucleoside triphosphate hydrolase protein
RNA Lariat Debranching Enzyme 1 (DRB1)	lariat debranching enzyme
Heat Shock Protein 90 (HSP90)	HSP90
Chromatin Remodeling Factor 1 (CLASSY1)	P-loop containing nucleoside triphosphate hydrolase protein

Figures

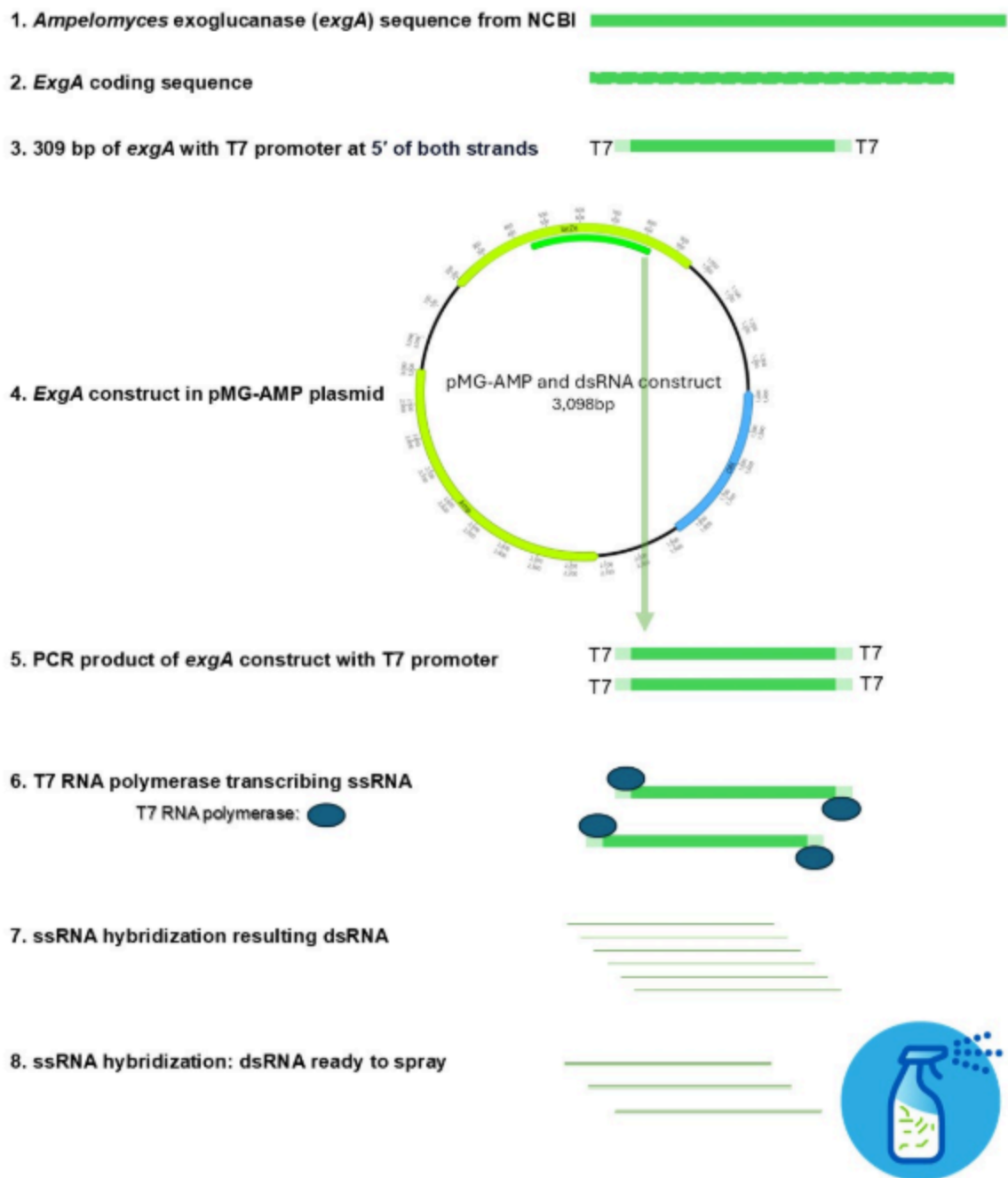


Figure 4

The steps of the design and synthesis of the dsRNA targeting the $\text{exo-}\beta\text{-1,3-glucanase}$ mRNA in a proof-of-concept Spray-Induced Gene Silencing (SIGS) experiment with *Ampelomyces* strain BRIP 72097.

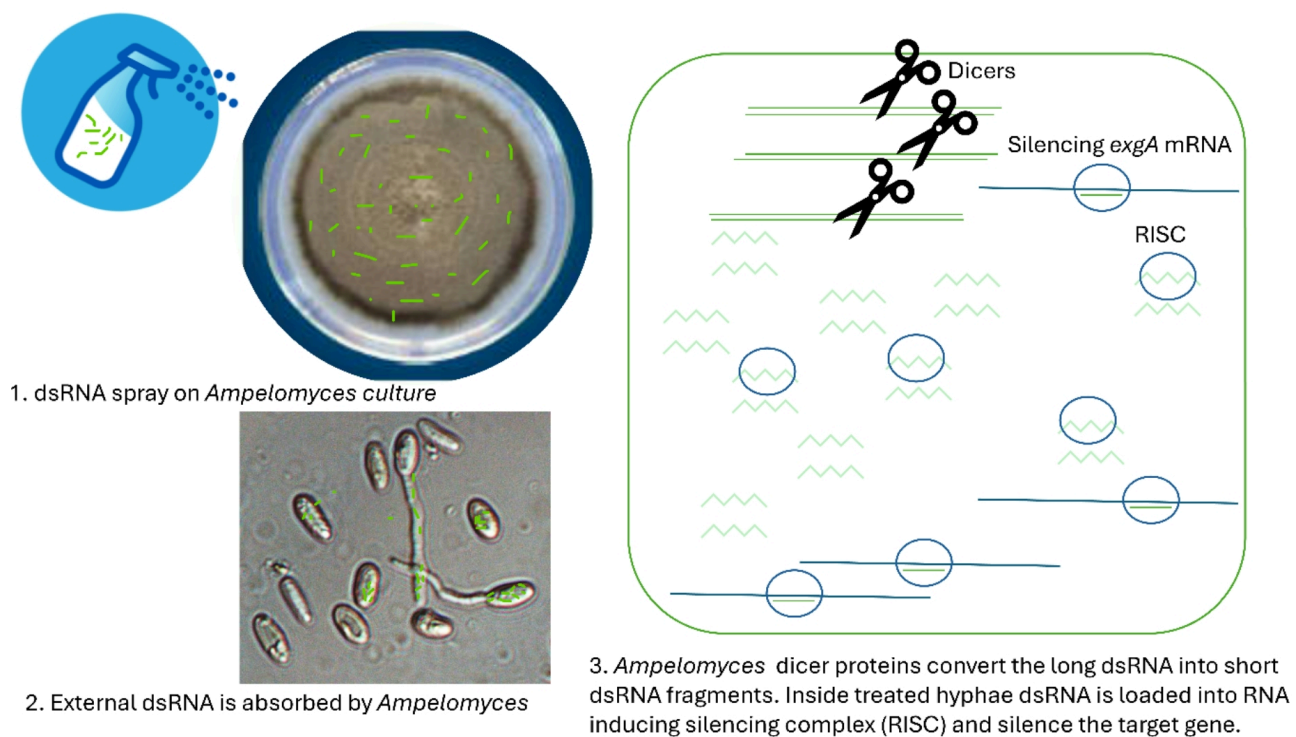


Figure 5

Hypothetic steps of the uptake and processing of the synthetic dsRNA during a proof-of-concept Spray-Induced Gene Silencing (SIGS) experiment with *Ampelomyces* strain BRIP 72097.

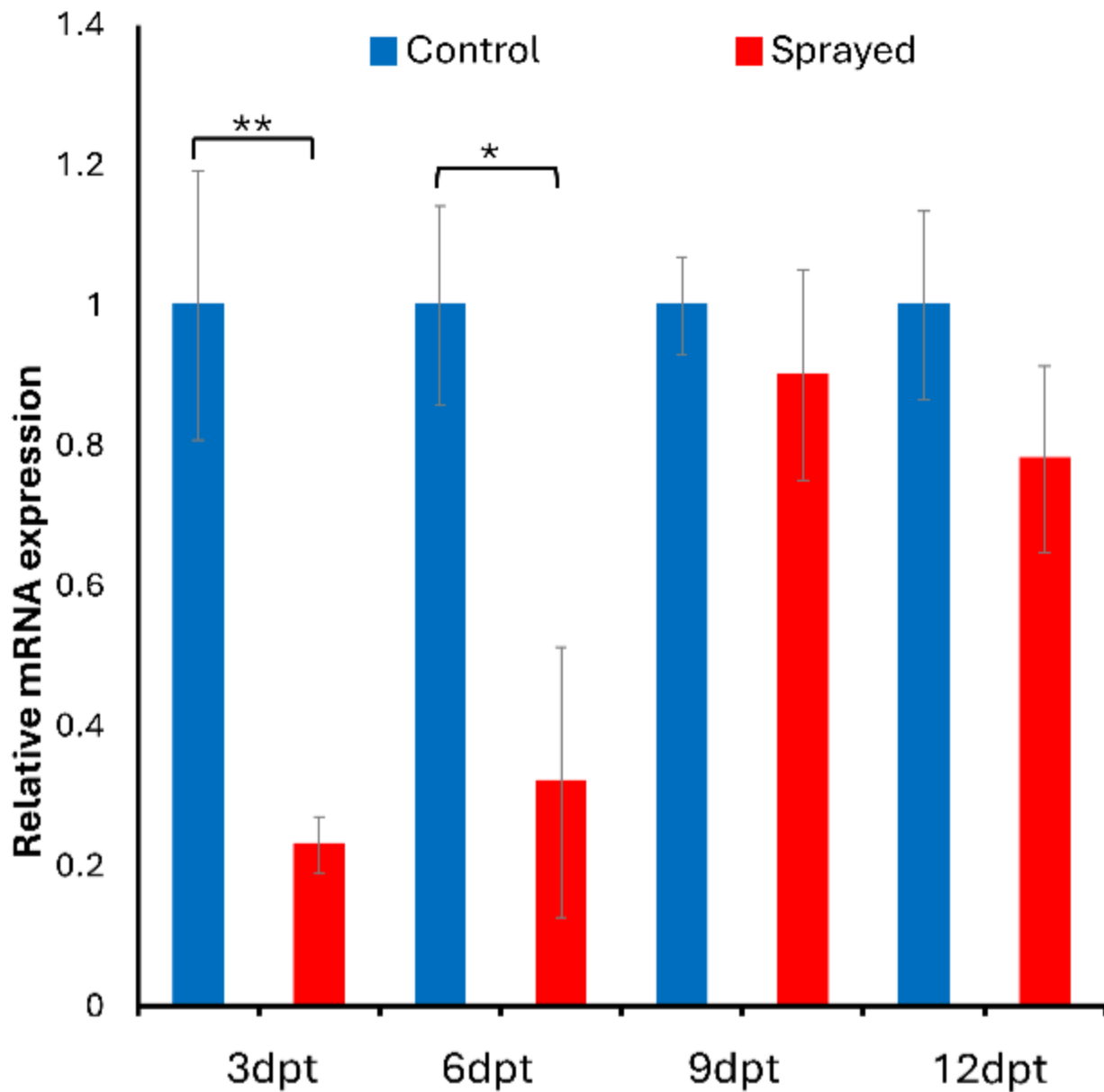


Figure 6

Relative exo-β-1,3-glucanase (*exgA*) mRNA expression three-, six-, nine- and 12-days post treatment (dpt) with dsRNA targeting *exgA*-mRNA. Significance levels are indicated as * $p < 0.05$ and ** $p < 0.01$.

Supplementary Files

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