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Harnessing RNA Interference to Constrain *Ustilago hordei* Growth and Barley Infection

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Abstract

Introduction: *Ustilago hordei* (*U. hordei*), a facultative biotrophic fungal pathogen, poses a significant threat to barley (*Hordeum vulgare*) crops, causing barley smut and substantial economic losses. Fungal colonies have a high risk of developing resistance to traditional fungicides upon usage and can pose potential risks to the environment. The survival and infection cycle of *U. hordei* is enabled through a number of genes suppressing the host immune system, including *UhAvr1*, *Fly1*, and the *beta-glucuronidase gene (GUS)*. This research project aims to explore the application of RNA interference (RNAi) to silence these genes implicated in barley colonization, aiming to understand the effect of Spray-Induced Gene Silencing (SIGS) using double-stranded RNA (dsRNA) on *U. hordei* growth.

Methods: Three pivotal genes (*UhAvr1*, *Fly1*, *GUS*) important to *U. hordei* infectivity were chosen to be amplified and transformed into dsRNA that contains small interfering RNA (siRNA) regions through in vitro expression in *Escherichia coli*. These genes were selected due to the roles they play in promoting *U. hordei* virulence and enhancing *U. hordei's* ability to metabolize complex carbohydrates. The impact of dsRNA on *U. hordei* growth will be assessed at concentrations of 50 ng/µL, 100 ng/µL, 150 ng/µL, 200 ng/µL. After growth is assessed, infectivity will be measured by comparing the number of barley plants infected versus the number of plants inoculated 6 days after inoculation.

Results: The application of target gene dsRNA will limit fungal growth and barley colonization. A positive relationship between translational inhibition and dsRNA concentration was observed in *U. hordei* populations. Furthermore, there was a negative relationship between the concentration of dsRNA applied to *U. hordei* and the fraction of plants infected versus inoculated.

Conclusion: Ultimately, this study provides insights into the application of the three potential dsRNAs in SIGS as a replacement for chemical fungicides with wide agricultural applications.

Keywords: ustilago hordei; barley smut; RNA interference; fungicide

Introduction

Ustilago hordei (U. hordei) is a facultative biotrophic fungal pathogen that primarily affects *Hordeum vulgare*, a worldwide economically and nutritionally important crop colloquially known as barley, causing a disease known as barley smut [1]. Barley smut is a common fungal disease that can lead to reduced yield and quality of barley crops and significant economic losses. *U. hordei* has a complex life cycle that involves both sexual and asexual stages that are coupled with its pathology [2]. These resting spores of *U. hordei*, named teliospores, act as the main markers of massive proliferation and are usually dispersed onto healthy barley, beginning the infection cycle [3, 4]. Currently, methods of disease management include crop rotation, using disease-resistant barley varieties, and fungicides [5]. However, the pollution resulting from fungicide usage and the preferential selection for fungicide-resistant *U. hordei* can pose serious long-lasting problems to the environment [5]. Recently, an RNA silencing approach known as Spray-Induced Gene Silencing (SIGS), which utilizes the nature of fungi to intake dsRNA upon exposure, provides a novel approach to combat fungal pathogens [6].

RNA interference (RNAi) is a natural sequence-specific gene regulation process using small RNA molecules and has become increasingly important as a tool for research in plants. The initiation of RNAi involves the cleavage of trigger RNA (dsRNA) into short interfering RNAs (siRNA) by the enzyme, Dicer. The siRNAs interact with the enzyme Argonaute (AGO) and are unwound into 2 single-stranded RNAs called sense and antisense RNA. The sense RNA is degraded by AGO, while the antisense RNA is incorporated into the RNA-inducing silencing complex (RISC), which allows the antisense RNA to hybridize with its complementary target mRNA [7]. This will lead to the

cleavage of target mRNA, resulting in target gene silencing. This research project aims to use RNAi to silence genes that are involved during colonization of barley and evaluate the effect of this treatment on *U. hordei* growth [8].

Several genes crucial to *U. hordei* pathogenicity were selected in this research project. Three genes, *UhAvr1, Fly1*, and the *beta-glucuronidase gene (GUS)* were chosen based on their functions and expression during barley infection. *UhAvr1* was selected as it actively expresses itself in the early stages of infection, leading to a general suppression in the host immune system [9]. The *Fly1* gene is present in both *U. hordei* and *Ustilago maydis*, the perpetrator of corn smut. It codes for fungalysin metalloprotease, which reduces plant chitinase activity [10]. Acting as the first line of defense, plant chitinase restricts fungal growth through fungal cell wall remodeling [11]. The expression of fungalysin metalloprotease, as a result, tears a hole in the airtight host defense mechanism, creating room for *U. hordei* survival. The third gene on the list, *GUS*, codes for the production of β-glucuronidase, a lysosomal enzyme responsible for breaking down complex carbohydrates in the host, influencing the structure of the barley cell wall [12]. Therefore, the *GUS* gene is also known to play an important role in barley colonization, and its product, β-glucuronidase, is produced in the tips of the hyphae of *Ustilago hordei* in infected barley tissue [13].

We hypothesize that the utilization of RNA interference with genes *UhAvr1, Fly1, GUS* is an effective mechanism in restricting the growth of *Ustilago hordei* and could potentially limit *U. hordei's* growth and ability to infect barley plants.

Methods

Culture Maintenance and Creating cDNA Library

The cultivation of *U. hordei* will be conducted in $YEPS_{light}$ (0.4% yeast extract, 0.4% peptone, and 2% saccharose). The culture will be maintained in liquid medium at 22 °C with continuous shaking at 200 rpm until the optimal density 600nm (OD_{600}) reaches 1.0 [1]. To produce a complementary DNA (cDNA) library for *U. hordei*, RNA will be extracted using a Masterpure™ Complete DNA and RNA Purification Kit (Epicentre®, Illumina®, MC89010). Genomic DNA is removed by the addition of ezDNase enzyme for 2 min at 37 ℃. ezDNase is inactivated under 55 ℃. To create cDNA strands using the RNA template, first mix 0.01 pg–0.5 µg template RNA with 0.5-1.0 µM of gene-specific primers and 0.5-1.0 mM of dNTPs. Add buffer (DTT+RNase inhibitor) and nuclease-free water to the reaction as well. When the mix is ready, add 1 µL of avian myeloblastosis virus (AMV) reverse transcriptase with 5 x AMV RT buffer to the solution; put the reaction mix under 42 ℃ for 60 minutes. To inactivate the enzyme, raise the temperature of the reaction mix to 70–85 °C for 5 to 15 min [14]. The cDNA products contain the *U. hordei* target genes that are essential for the colonization of barley that will be amplified using polymerase chain reaction (PCR) and used as the template for the synthesis of dsRNA in later steps [15].

Choosing the siRNA for the Genes of Interest

The sidirect database will be used in the selection of suitable siRNA for the target genes *UhAvr1, Fly1, GUS*. siRNAs are often 20-30 nucleotides in length [16]. The database can identify the effective regions for siRNA of target genes. For our project, we will identify three effective areas for each gene. These regions help build effective siRNA and prevent excessive off-target trial-and-error [17].

dsRNA Synthesis

After the creation of the cDNA library for *Ustilago hordei* and the selection of target genes, PCR and primers specific to the siRNA of *UhAvr1, Fly1*, and *GUS* are used to amplify the genes of interest. The target gene amplicons are purified and collected through gel elution using QIAquick Gel Extraction Kit (QIAGEN, 28704). This project will use engineered bacterial expression to produce dsRNA. The bacterial strain selected is *Escherichia. coli (E. coli)* HT115 due to its familiar genetic background, ease of genetic manipulation, and lack of RNase III. A lack of RNase III is important to prevent the degradation of dsRNA, our final product. The vector we chose is L4440 with oppositely oriented T7 promoters, a DNA sequence that allows T7 RNA polymerase (T7 RNAP) to recognize and initiate transcription, due to its reliable presence in literature and widespread use in other studies. T7 RNAP will be synthesized on induction by isopropyl β-D-1-thiogalactopyranoside (IPTG), which will synthesize complementary strands of RNA, forming dsRNA [18]. Gel electrophoresis will be used to confirm the presence of dsRNA. A band of the corresponding size will be observed if our target dsRNA is on the gel. The product is purified using ion pair reverse-phase high-performance liquid chromatography [19].

Testing the Growth Rate and Infectivity After dsRNA Intake

Following the extraction of the dsRNA from *E. coli*, the liquid culture medium of *U. hordei* will be treated at 50 ng/µL, 100 ng/µL, 150 ng/µL, 200 ng/µL of dsRNA [16]. Corresponding control groups (SS20 Buffer of the same volume only) for each treatment group will also be included in the experimental setup. This project will incubate the culture for 12 hrs and 24 hrs, then measure the OD_{600} of *U. hordei* in distilled water to quantify *U. hordei* growth [1]. We will use 1:1 solution of distilled water and $YEPS_{light}$ as a blank to which other values will be compared relatively. If there is the presence of viable *U. hordei* after dsRNA treatment, these colonies will be exposed to barley to test for infectivity. Pathogenicity was measured by comparing the number of barley plants infected versus the number of plants inoculated 6 days after inoculation. Infected plants will show visible black spots on the barley seeds [20].

Results

This project expects that cell culture will produce viable *U. hordei* fungal colonies that express the target gene proteins (*UhAcr1*, *Fly1*, *GUS*). A complete cDNA library is expected to be created based on the transcriptome of *U. hordei*. The PCR on cDNA for the three target genes will amplify the target gene sequence substantially. These cDNA fragments act as the precursor for the production of dsRNA, which contains the potential siRNA effector sequences obtained from the sidirect database search. This project expects the target gene dsRNA product, when applied to the *U. hordei* culture, to limit fungal growth and colonization of barley plants. More specifically, it is expected that there will be a positive relationship between the effect of translational inhibition on *U. hordei* exhibited by dsRNA treatment and the concentration of dsRNA. The *U. hordei*, if viable colonies exist after dsRNA treatment, is anticipated to decrease its infectivity. In other words, the higher the concentration of dsRNA, the lower the growth of *U. hordei* in liquid medium, and fewer infected barley plants will be observed.

Conclusions

This research project provides new insights into RNAi as an alternative to chemical fungicides and is a potential antifungal method in the face of rising antifungal resistance. Using SIGS, dsRNA of the three genes that we identified (*UhAcr1*, *Fly1*, *GUS*), can be applied in a convenient manner. Therefore, considering these expected results, our research can contribute to the field of agriculture by identifying alternative or supporting solutions to chemical fungicides.

List of Abbreviations Used

GUS: *Beta-glucuronidase gene*
RNAi: RNA interference
siRNA: Small interfering RNA
dsRNA: double stranded RNA
SIGS: spray-induced gene silencing
AGO: argonaute
RISC: RNA-induced silencing complex
cDNA: complementary DNA
OD: optimal density
$YEPS_{light}$: 0.4% yeast extract, 0.4% peptone, and 2% saccharose solution
PCR: polymerase chain reaction
IPTG: isopropyl β-D-1-thiogalactopyranoside

Conflicts of Interest

The author(s) declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

No animals or human subjects were used or studied in the making of this research proposal project. Therefore, no ethics approval nor participant consent is necessary.

Authors' Contributions

EJ: made substantial contributions to the design of the study, reviewed literature, drafted the manuscript, and gave final approval of the version to be published. Is in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
OX: made substantial contributions to the design of the study, reviewed literature, drafted the manuscript, and gave final approval of the version to be published. Is in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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