PROJECT SUMMARY

Instructions:
The summary is limited to 250 words. The names and affiliated organizations of all Project Directors/Principal Investigators (PD/PI) should be listed in addition to the title of the project. The summary should be a self-contained, specific description of the activity to be undertaken and should focus on: overall project goal(s) and supporting objectives; plans to accomplish project goal(s); and relevance of the project to the goals of the program. The importance of a concise, informative Project Summary cannot be overemphasized.

Title: Resilience And Optimization Of Rnai In Fungal Disease Management.

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CO-PD: PD/PI 2 Name (Last, First, MI)	**Institution:**
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CO-PD: PD/PI 7 Name (Last, First, MI)	**Institution:**

Fungi are the most economically devastating class of plant pathogens and affect a wide range of crops critical to global food security. RNA interference (RNAi) has emerged as a promising technology to combat fungal diseases, but substantial knowledge gaps in application limit its usage. This project will address critical questions surrounding the intraspecies susceptibility of S. sclerotiorum to RNAi and the durability of this technology across multiple pathogen generations. To achieve this, we will use a large collection of isolates with robust genetic, geographic and host range diversity. This collection will be used to challenge transgenic soybeans targeting critical virulence genes through host induced gene silencing (HIGS) and both the ubiquity and mechanistic basis of RNAi insensitivity in the collection will be evaluated. Simultaneously, multiple isolates will be used to sequentially infect resistant HIGS lines and select for isolates capable of breaking RNAi-mediated resistance, thus generating critical data on the resilience and molecular "weak points" of this technology. Finally, to investigate novel uses of RNAi, we will validate the usage of tRNA-like motifs as a possible approach to increase dsRNA systemic activity and control fungal diseases without the regulatory burden of traditional GMOs. In the future this work will guide the integration of RNAi into current IPM practices and lead to the development of highly systemic dsRNA technology to facilitate environmentally sustainable control of fungal diseases.

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Title: Resilience and Optimization of RNAi in Fungal Disease Management

Introduction

Transgenic plants capable of suppressing fungal disease through RNA interference (RNAi) have become an attractive approach in the field of pathogen management, as these plants can be tailor made to target a fungal pathogen of interest with limited off-target environmental effects[1–3]. Despite this promise, RNAi technologies suffer from substantial knowledge gaps that limit their widespread agricultural adoption. While the molecular mechanisms facilitating RNAi are nearly ubiquitous within the fungal kingdom, some species appear to either lack this machinery (e.g. *Ustilago maydis*[4]) or do not absorb externally applied double stranded RNA (dsRNA), rendering the technology inert (e.g. *Zymoseptoria tritici*[5]). Because of the difficulty and expense in demonstrating dsRNA uptake and silencing, these "proof-of-concept" assays are typically only performed on a single lab strain of the pathogen. This practice, while economical, ignores the large and intrinsic variation that is observed in wild fungal populations. Within these populations, researchers commonly observe vast differences across nearly every measurable phenotype, including fungicide resistance, virulence, stress tolerance, and secondary metabolism, so it is unwise to assume that a population's susceptibility to exogenous dsRNA would be homogeneous. Such species level differences in response to RNAi have already been observed in the pathogenic fungus *Colletotrichum gloeosporioides*[6,7].

To address questions surrounding intrapopulation sensitivity to RNAi, we will investigate *Sclerotinia sclerotiorum*, which is the causative agent of Sclerotinia Stem Rot (SSR) on a range of dicotyledonous crops worldwide and has a demonstrated susceptibility to RNAi-mediated disease management[6,8]. **In Objective 1, we will employ a large collection of *S. sclerotiorum* isolates to assess inter-isolate variation in sensitivity to RNAi.** This collection was gathered from diverse (n= 25) hosts found across multiple regions (n = 30) in the United States (Figure 1). This collection is maintained by our collaborator, Dr. Richard Webster at North Dakota State University, and most isolates have been screened for phenotypic variation and haplotype[9]. As opposed to the costly, in-vitro synthesized dsRNA, we have worked with our collaborator Dr. Mehdi Kabbage at UW Madison to develop transgenic soybeans utilizing host-induced gene silencing (HIGS) to target the *S. sclerotiorum* genes *Ssoah1* or *Sslac2*. These genes have been previously demonstrated to be both critical for *S. sclerotiorum* virulence and can be efficiently targeted through plant produced dsRNA[8,10]. The successful uptake of dsRNA and silencing of these genes translates to greater SSR resistance in HIGS plants when compared to non-transformed controls, making the infection of these lines an efficient proxy for RNAi efficacy.

A second major unknown surrounding the use of RNAi in fungal disease management is the potential for resistance development within fungal populations continuously exposed to lethal gene silencing. It is well established that fungi develop resistance to chemical fungicides, often through overexpression of, or mutations in, the fungicide target site[11]. Despite this, the putative mechanisms used by fungi to overcome gene silencing are entirely unexplored. It is unlikely that RNAi will prove to be a silver bullet providing permanent control of fungal diseases and it is therefore the focus of **Objective 2 to explore the question of resistance development through an accelerated selection approach**. To achieve this, three *S. sclerotiorum* isolates from distinct hosts, geographies, and haplotypes will be used for a continuous challenge experiment on the two transgenic lines described above. A convenient feature of *S. sclerotiorum* is the rapid cycling between generations, with the capacity to fully transition from plant infection, to overwintering sclerotia, and back to infectious hyphae in ~14 days. By continuously infecting moderately

resistant plants, we will provide strong selective pressure on these strains to overcome this resistance, with the goal of this objective being the identification of one or more resistance-breaking mechanisms.

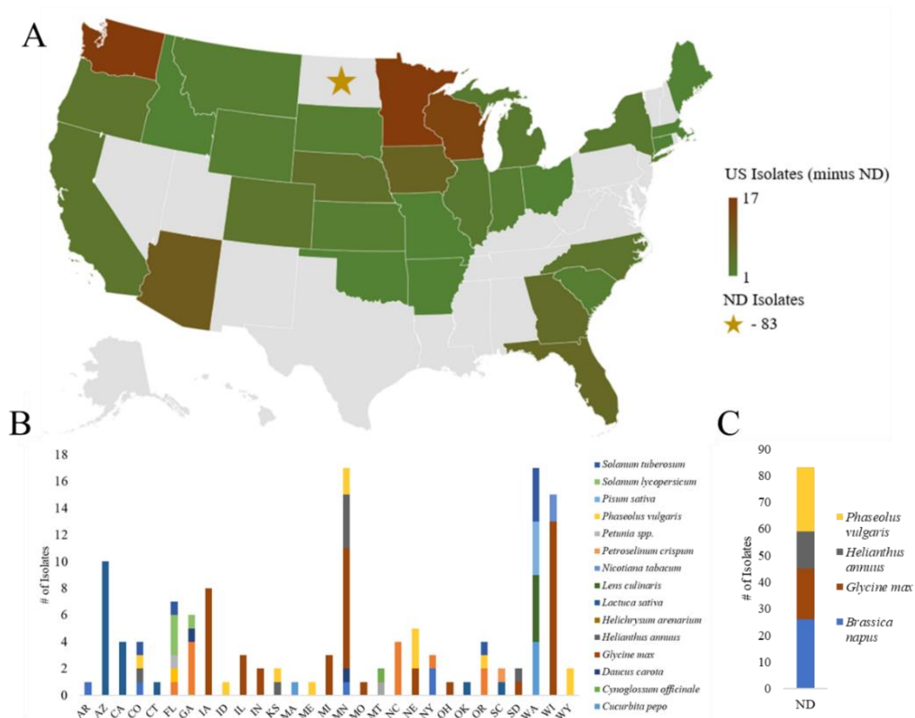

Figure 1. Summary of *S. sclerotiorum* population used in this study. A) Map of the contiguous US demonstrating the relative contribution of each state to the collection. B) Host breakdown by state C) Host breakdown of isolates collected in North Dakota (ND).

While it's critical to identify the functional limitations of RNAi in disease management, previous work by multiple groups have demonstrated that it is a potentially valuable tool in an integrated IPM program to control fungal diseases[6,8]. The most broadly applicable version of this technology is spray-induced gene silencing (SIGS), which applies dsRNA as a foliar spray to both be taken up by the plant and act directly on fungal pathogens. Research in this field has focused largely on the environmental stability of dsRNA, often through coating with nanomaterials, but the systemic movement of these molecules through plant tissue is also critical for their efficacy[12]. Typically, systemic activity of fungicides is an intrinsic feature of the molecule and difficult to manipulate, but advances in our understanding of RNA biology have demonstrated that the addition of transfer RNA (tRNA)-like motifs to RNA molecules can drastically improve their systemic movement in plants[13,14]. Given these features, the focus of **Objective 3 is to optimize the movement of dsRNA molecules through modification with tRNA-like motifs, and evaluate the effect of these alterations on cross kingdom RNAi efficacy.** To achieve this, *in-vitro* synthesized dsRNA will be generated with and without the 3' addition of multiple tRNA-like motifs. These will be applied to multiple *S. sclerotiorum* host species, including Arabidopsis, *Nicotiana benthamiana,* and soybean, to evaluate the effect that these formulations have on both local and distal disease susceptibility. Moreover, we will estimate the effect that these alterations have on the movement and RNAi efficiency within and across fungal cells using GFP-expressing mutants of *S. sclerotiorum*. In all of these evaluations we will be capable of measuring the movement/activity of these molecules using RT-qPCR and the downstream effect of the gene silencing through either lesion measurements (*in planta*) or the quantification of GFP fluorescence (*in funga*).

Rational and Significance

Recent years have shown an explosion of interest in RNAi-based control of pests and pathogens, with successes in control of Colorado Potato Beetle and Western Corn Rootworm through spray-induced gene silencing and host-induced gene silencing, respectively[15,16]. The usage of these technologies to control fungal disease including, but not limited to, SSR is an area of active technology development despite substantial unknowns. This proposal will address both questions surrounding the molecular mechanisms involved in fungal response to RNAi-mediated disease management and many practical concerns surrounding its utilization. This proposal directly addresses the A1112 program priority E by investigating mechanisms of pest resistance to pesticides or toxins in genetically modified plants and helping to develop strategies to mitigate resistance.

Approach

OBJECTIVE 1– Characterization of Intrapopulation Differences in *S. sclerotiorum* Susceptibility to RNAi

To evaluate the functional response to RNAi within a fungal population, we will utilize a large population of the broad-host range pathogen *S. sclerotiorum* containing 222 isolates, collected from 30 states, and spanning 16 distinct hosts (Figure 1). Despite the relatively clonal nature of *S. sclerotiorum*, a high level of genetic diversity has been observed across populations and differences in isolate aggressiveness and fungicide sensitivity within these populations is common[9,17–19].

Objective 1a – Screening of population aggressiveness against WT and HIGS soybean

To assess the susceptibility of this population to RNAi-mediated fungal disease management, we will measure the aggressiveness of each isolate on two transgenic lines targeting the *S. sclerotiorum* genes *Ssoah1* or *Ssclac2* through host-induced gene silencing (HIGS). These lines were generated by the Wisconsin Crop Innovation Center and the selected genes have previously been demonstrated to provide significant resistance when targeted through virus-induced gene silencing[8,10]. Additionally, both genes demonstrate high levels of sequence conservation across all sequenced *S. sclerotiorum* isolates (data not shown). Soybeans from each of the two transgenic lines and an untransformed Williams 82 soybean (the genetic background of both lines) will be inoculated with each isolate and lesions will be measured as previously described[20].

Objective 1b – Characterization of RNAi Resistant Isolates

Isolates with minimal differences in lesion size between transgenic lines and untransformed controls will be further evaluated to determine the mechanism of RNAi resistance. The primary mechanisms to be evaluated are: 1) mutations in the target genes, 2) failure by the isolate to take up exogenous dsRNA, 3) lack of canonical RNA silencing occurring in the isolate.

To assess possible mutations, the target genes will be amplified from the tested isolate and sequenced to identify mutations that would undermine gene silencing efficacy. If this was the case, we would additionally expect to see differences in aggressiveness between the two transgenic lines as it's unlikely that both targets would be the subject of mutations severe enough to undermine RNAi. **To investigate a failure of uptake**, we will expose the isolates to in-vitro synthesized dsRNA tagged with a Cy3 fluorophore. This procedure has been used to demonstrate dsRNA uptake in the *S. sclerotiorum* lab strain 1980 and this strain would be used as a control in these experiments[8]. By quantifying fluorescence, we can evaluate the relative capacity of these isolates to actively absorb exogenous dsRNA and compare this activity to a known control.

Finally, **to examine a loss of RNA silencing,** we will use real-time PCR to measure gene expression of the targeted genes grown on potato dextrose agar (PDA) alone and in the presence of in-vitro synthesized dsRNA targeting both genes. The dsRNA sequence will be designed to match the ~350 bp segment being employed in HIGS plants and gene silencing efficiency will be compared to strain 1980. Both genes are constitutively expressed on PDA, allowing for a simple in-vitro assay without the need for plant infection[8,10]. General phenotypic characteristics including growth, sclerotial formation, and resistance to abiotic stress will be evaluated in RNAi insensitive isolates to identify possible fitness penalties observed in this experiment.

Expected outcomes

Objective 1 will identify isolates capable of overcoming the RNAi-mediated resistance present in our HIGS soybean lines. Furthermore, the mechanistic diversity of this "resistance breaking" will be characterized to determine possible fitness costs, cross-resistance to multiple HIGS targets, and scalability of the technology. While we will assess the expected mechanisms that would lead to dsRNA insensitivity, given the wide diversity of isolates we may additionally identify mechanisms distinct from those identified in other fungal organisms.

Potential problems and alternative approaches

While the collection being used for this experiment is diverse, we cannot be absolutely confident that any given isolate will be fully virulent against our HIGS lines. Although the failure to identify such an isolate would negate Objective 1b, it would provide unexpectedly robust evidence for the efficacy of RNAi in the disease management of *S. sclerotiorum* and possibly other fungal plant pathogens as well. As the collection being provided by our collaborator Dr. Richard Webster has been well characterized previously there are no expected issues concerning the *S. sclerotiorum* isolates. The project director Dr. Nathaniel Westrick has extensive experience with *S. sclerotiorum* virulence assays and all of the proposed molecular techniques described in 1b from his time working in the lab of Dr. Mehdi Kabbage at the University of Wisconsin – Madison, so there are no predicted issues.

OBJECTIVE 2– Using Accelerated Selection to Examine Future Fungal Resistance to RNAi

No disease management tool in history has proven itself a perfect solution, and given the increasing interest in RNAi-mediated resistance, it's extremely likely that fungal pathogens will evolve mechanisms to overcome these tools just as they have for chemical fungicides many times before[11]. Unfortunately, while extensive studies have documented the mechanisms used by pathogens to develop fungicide resistance, it is entirely unknown what mechanisms will be used by fungi to combat RNAi. To address this gap, we will use an accelerated selection experiment to rapidly cycle multiple isolates of *S. sclerotiorum* through the previously described HIGS soybean lines to impose strong selective pressure on these strains. Similar approaches for generating fungicide resistant isolates of *S. sclerotiorum* and closely related fungi have been used in the past, suggesting that the species is amenable to such an approach[19,21].

Objective 2a – Continuous infection of RNAi Resistant Soybeans with *S. sclerotiorum* isolates

Three isolates of *S. sclerotiorum* with distinct hosts, geographies, and haplotypes have been selected from our collection and will be inoculated on cut petioles of the first trifoliate of *Ssoah1* or *Sslac2* HIGS lines under three different scenarios (Figure 2). In scenarios A and B, these three isolates will be inoculated on three distinct soybeans of either HIGS line and allowed to infect for 10 days. Either sclerotia or sclerotial initials will be recovered from the infected soybeans with the largest lesions and germinated on PDA plates in order to repeat the infection.

In every generation lesion size will be measured to record the progress of the pathogens in overcoming plant resistance and only the largest lesions will be selected to be used on the following generation. This experiment will be conducted for at least 30 generations, with an expected generation time of 14 days between inoculations. In scenario C, the three isolates will be alternated between each of the two HIGS lines in each generation. This will be done both to evaluate the efficacy of rotations between HIGS targets and to provide a stronger selective pressure on the pathogen's dsRNA uptake and RNA silencing machinery, rather than mutations in *Ssoah1* or *Sslac2*. A benefit of *S. sclerotiorum* as a model to study this mechanism is that it does not sporulate under typical laboratory conditions and therefore the risk of isolates with increased resistance to RNAi escaping containment is minimal.

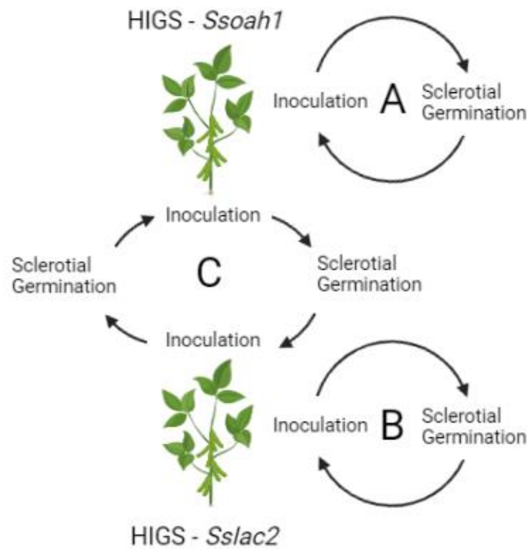

Figure 2. Experimental setup for accelerated evolution experiment.

Objective 2b – Characterization of RNAi Resistant Isolates

Isolates with significantly increased lesion sizes will be evaluated for their mechanism to overcome RNAi-mediated resistance using the same methodology described in Objective 1b. The primary mechanisms to be evaluated are: 1) mutations in the target genes, 2) failure by the isolate to take up exogenous dsRNA, 3) lack of canonical RNA silencing occurring in the isolate. We will additionally perform whole genome sequencing (a combination of PacBio and Illumina) on hypervirulent isolates and freezer stocks of the same isolates generated prior to the experiment. Comparative genomics will be used to identify mutations with a possible role in decreased susceptibility to dsRNA.

Expected outcomes

Objective 2 will induce strong selective pressure on multiple isolates of *S. sclerotiorum* to overcome RNAi-mediated resistance and we will collect data on both the durability of this resistance across multiple generations and the molecular changes needed to overcome it. Additionally, we will generate data on the relative value of "RNAi target rotation" as a strategy to increase the durability of HIGS plants under field conditions.

Potential problems and alternative approaches

The potential complications of Objective 2 largely mirror Objective 1, in that there is a possibility that after 30 passages through resistant plants, no significant increase in virulence will be observed. While this would be surprising, no experimental data on serial passaging of *S. sclerotinia* has been published and is possible. Similar to objective 1, however, such a result would be a valuable finding as it would demonstrate the durability of this technology across multiple generations of pathogen infection, each of which would mirror a field season, as *S. sclerotiorum* is a monocyclic pathogen. Although this experimental design would not include the production of ascospores, which are involved in many infections, it would effectively imitate the myceliogenic germination of sclerotia which drives basal stalk rots in a number of hosts, such as sunflower. In the case that no hypervirulent isolates are generated, whole genome sequencing will instead be performed on strains with diverse morphologies/ virulence characteristics identified in Objective 1.

OBJECTIVE 3– Optimizing the systemic movement of dsRNA targeting *S. sclerotiorum*

While the bulk of this proposal seeks to place guard rails on the usage of RNAi in disease management and better understand the resiliency of the technology, previous work suggests that it can be a valuable tool in fighting fungal diseases when used correctly[6,8]. The efficacy of this technology depends on multiple factors, including the criticality of the gene being targeted, the stability of the molecule on the plant, and the systemic uptake and movement of the molecule after being sprayed[22]. Current research has focused extensively on the first to priorities, but relatively little attention has been paid to optimizing its systemic movement, so this objective will seek to optimize this activity of exogenously applied dsRNA through the addition of tRNA-like motifs. These hairpin sections of RNA have been demonstrated in other plant systems to dramatically increase the systemic movement of RNA molecules through plant phloem when amended to typically non-systemic mRNA[13,14].

Objective 3a – Assess the translocation of modified dsRNA within plant tissue

Double-stranded RNA sequences targeting *Sslac2* will be generated with and without distinct hairpin structures on their 3' end and applied directly to plant tissue. To assess the directional systemic movement of the synthesized dsRNA molecules, they will be sprayed on the apical and basal leaves of Arabidopsis, *Nicotiana benthamiana,* and soybean plants (Figure 3). *Sslac2* was chosen for this experiment as our work has demonstrated that it is critical for infection of *N. benthamiana*, unlike other virulence genes such as *Ssoah1*[10]. The presence of these molecules in distal leaves will be assessed using RT-qPCR for full length dsRNA and stem-loop RT-qPCR for siRNA. To validate that translocated dsRNA translates to disease control, disease assays will be conducted using *S. sclerotiorum* 1980 on both directly sprayed and distal leaves (Figure 3).

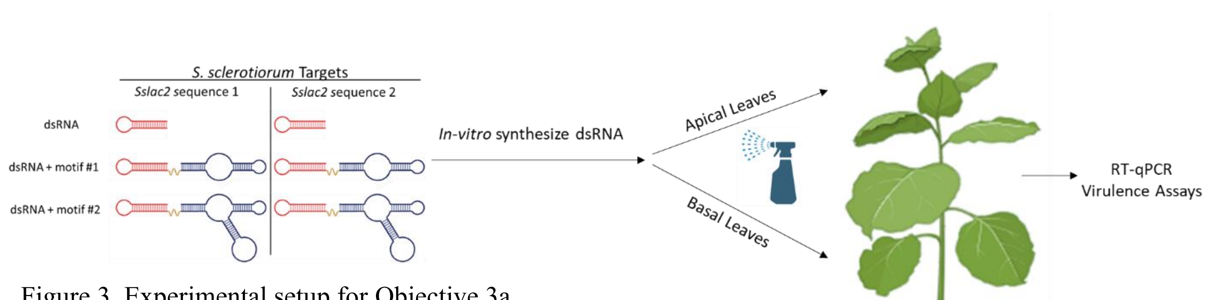

Figure 3. Experimental setup for Objective 3a.

Objective 3b – Assess the activity of modified dsRNA in silencing *S. sclerotiorum* genes

To confirm that these modifications will not undermine the efficacy of dsRNA in silencing target genes, GFP expressing cultures of *S. sclerotiorum* will be directly spiked with GFP-targeting dsRNA molecules with the above-described modifications (Fig. 3). This strain was produced by Dr. Jeff Rollins at University of Florida and has already been assessed for GFP expression in our lab. Confocal fluorescent microscopy measuring GFP expression and RT-qPCR measuring gene expression will be used to assess the efficacy of dsRNA in both local and distal tissue (Fig 4.). The assay will be conducted on

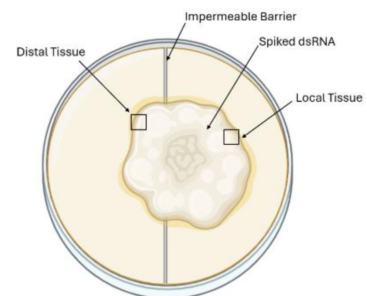

Figure 4. Experimental setup for Objective 3b.

cultures grown over split-plate petri dishes with an impermeable central barrier. This will allow us to isolate the effect of dsRNA dissolution through the media from translocation within fungal tissue.

Expected outcomes

Objective 3 will yield critical data about the structural features of dsRNA which facilitate systemic movement within plants to optimize disease management. It will directly inform modifications which could potentially be translated into RNAi technologies currently being developed.

Potential problems and alternative approaches

This objective focusses entirely on preliminary knowledge which has been well validated by other groups and techniques which the Westrick lab has extensive experience. As always, a null result demonstrating no alteration in systemic movement is possible, but that result would be inherently valuable for future SIGS application work.

<u>Summary and Future Directions</u>

Fungi are the most economically devastating class of plant pathogens and affect a wide range of crops critical to global food security[23]. RNAi has emerged as a promising technology to combat fungal diseases, but substantial knowledge gaps in application limit its usage. This project will address critical questions surrounding the intraspecies susceptibility of *S. sclerotiorum* to RNAi and the durability of this technology across multiple pathogen generations. Additionally, we will validate possible approaches to optimize dsRNA movement as a possible approach to control fungal diseases without the regulatory burden of traditional GMOs. In the future this work will guide the integration of RNAi into current IPM practices and refocus current RNAi disease management strategies around optimized systemic activity of the applied dsRNA.

Project Timetable	Year 1				Year 2			
Objective 1a: Screen *S. sclerotiorum* for RNAi Sensitivity	■	■	■	■	■			
Objective 1b: Mechnistic Characterization of Insensitive Isolates					■	■	■	■
Objective 2a: Serial Passaging of Isolates	■	■	■	■	■			
Objective 2b: Mechanistic Characterization of Insensitive Isolates					■	■	■	■
Objective 3a: Translocation of Modified dsRNA Within Plant Tissue	■	■	■	■	■			
Objective 3b: Activity of Modified dsRNA in Silencing *S. sclerotiorum*						■	■	■

<u>Response to Reviewer Comments</u>

The proposal was submitted in the 2023 RFA cycle and was positively reviewed by reviewers, but objective 3 was initially viewed as too ambitious. The initial iteration of objective 3 was to study systemic activity of RNAi by generating transgenic *N. benthamiana* that expressed dsRNA molecules with the motifs mentioned in Fig. 3 and assess their root-to-shoot movement through graft junctions. Reviewers were concerned that the timeline for generating that many transgenic plants was too tight, so the objective was altered to focus on in-vitro synthesized dsRNA which could be synthesized quickly. In the original iteration it was also proposed that the isolates to be used in Objective 2A would be chosen during Objective 1, but that has been updated to clarify that they have already been chosen.

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