Project FY22-NW-002: Conventional and Marker-based Breeding to Improve Yield and FHB Resistance in Wheat

---

[During the reporting period May 1, 2024 - April 30, 2025, we have advanced our studies and completed a PhD desertation. The PhD student graduated in November 2024, and to this end has two peer reviewed papers published and one under review in descent scientific journal outlets. We have appended our activities and results in the end of the Project report we provided last year for FY22-TS-009: Biodegradable Nanomaterial-based Non-GMO RNAi Delivery for Controlling FHB Disease].

1. What are the major goals and objectives of the research project?

Spray-Induced Gene Silencing (SIGS) can be used as a safe and robust method of plant protection for both pre-harvest (Koch et al., 2016; Mitter et al., 2017) and post-harvest strategy (Wang et al., 2016), which is especially also useful for FHB disease with continuous mycotoxin production after harvest. In this research, which is a collaboration between plant-fungal scientists and a nanotechnologist, the goal is to use principles of RNA interference (RNAi) (Agrawal et al., 2003) and host-induced gene silencing (HIGS) (Huang et al., 2006) to develop non-transgenic approaches for wheat resistance to FHB. In particular, this research aims to test the hypothesis that whether a spray product, containing double-stranded RNA tool(s), can control the FHB disease. Because the lifetime of RNA molecules in the environment is short, and they are degraded rapidly, we aim to leverage nanotechnology and incorporate it into silencing RNA tools for slow-release and possibly the penetration of RNA molecules to plant or fungal cells.

2. What was accomplished under these goals or objectives? *(For each major goal/objective, address these three items below.)*

What were the major activities?

The Chitosan Nanoparticles (ChNPs): The ChNPs used in this study were prepared using ionic interaction by cross-linking the positively charged amino groups of chitosan with negative charge phosphate groups of tripolyphosphate (TPP). During the preparation of nanoparticles, the ratio of chitosan-TPP was optimized to attain the nano-size positive charged particles. Therefore, the final chitosan-TPP ratio of 5:1 was selected for the preparation of positively charged ChNPs and further siRNA loading. The siRNA loaded ChNPs were developed using previously reported ionic gelation method used for the preparation of peptides, protein, and drug molecule nanoparticles. However, despite the effective synthesis of the nanoparticles and siRNA attachment the ChNPs by itself showed anti-fungal properties which did not provide the effective comparison between control studies with and without the siRNA.

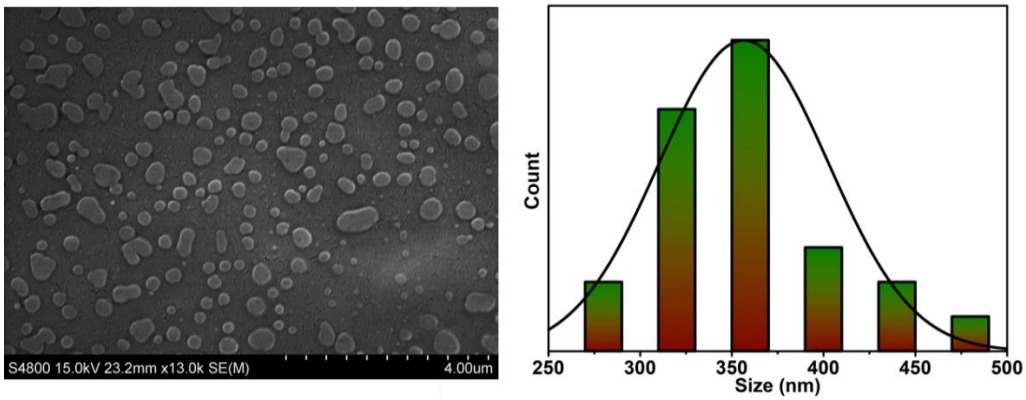

Figure 1. FESEM image and size distribution histogram of as-prepared siRNA loaded ChNPs.

For this issue, the team replace the synthesis of ChNPs with graphene quantum dot nano particles as an effective alternative without anti-fungal properties. The Graphene Quantum Dots (GQDs) used in this study were prepared (Figure 2a) using the previously reported method with minor modifications through a bottom-up approach by direct pyrolysis of citric acid (CA). The size and shape of GQDs were confirmed using Transmission Electron Microscopy (TEM) and the corresponding TEM image is shown in Figure 2b. The presence of several nanosized black dots (indicated with yellow color arrows) in the TEM image confirms the successful synthesis of GQDs having a size of around 2-5 nm. Since the siRNA is a negatively charged molecule, to load the siRNA onto GQDs, it is necessary to understand the surface charge of as-synthesized GQDs. Therefore, the zeta potential of GQDs was collected using Zetasizer and observed that GQDs displayed (Figure 2c) a slightly negative charge (-1.71 mV). To make the surface charge of GQDs positive, the chitosan surface functionalization strategy was used. The zeta potential collected (Figure 2d) after the surface functionalization of GQDs with chitosan exhibited a positive surface charge (+59.1 mV). These surface functionalized GQDs were further used for loading of siRNA through the ionic interaction mechanism and subjected to further characterization.

1b. Integration of designed dsRNA into core shell nanoparticles with uniform spray application process.

The team has identified appropriate process to embed siRNA onto GQDs. the next steps would be to identify appropriate process of administering siRNA functionalized GQDs onto plants using spray or syringe injection. This will be part of the next year's effort on the project.

Objective 2. Evaluate silencing of candidate genes in *F. graminearum* with siRNA oligos delivered by nanoparticles

2a. Silencing of candidate genes with nanoparticle-coated siRNA oligos.

We first designed and ordered two siRNA oligos targeting the *MGV1* and *COT1* genes that are critical for vegetative growth and plant infection. When treated with 20 mM siRNA oligo of *MGV1*

and *COT1* coated with chitosan nanoparticles, conidia of *F. graminearum* failed to germinate. However, the blank controls with chitosan nanoparticles alone also displayed inhibitory effects on conidium germination in *F. graminearum*. Therefore, we decided to switch to Graphene Quantum Dots (GQDs) as nanoparticles to treat siRNA oligos. When treated with 25% *MGV1* siRNA oligo coated with GQDs, conidium germination rate was 2.1% at 6 h. Under the same conditions, conidium germination rate was 81.7% in the blank GQDs control. For *COT1*, treatments with 25% siRNA oligo reduced over 60% germination rate at 6 h but treatments with 50% siRNA oligo blocked germination. The siRNA oligos targeting *GPMK1* and *FgHOG1* have been designed and ordered (back ordered). The *GPMK1* oligo will be used to testing whether treatments with siRNA oligos targeting GPMK1 that is important for plant infection but dispensable for hyphal growth will reduce plant infection. To determine whether the silencing effects of GQDs coated siRNA oligos are not unique to *F. graminearum*, we tested silencing the PMK1 MAP kinase gene that is essential for appressorium formation in the rice blast fungus. Treatments with GQDs coated PMK1 siRNA oligo were inhibitory to appressorium formation in the rice blast fungus.

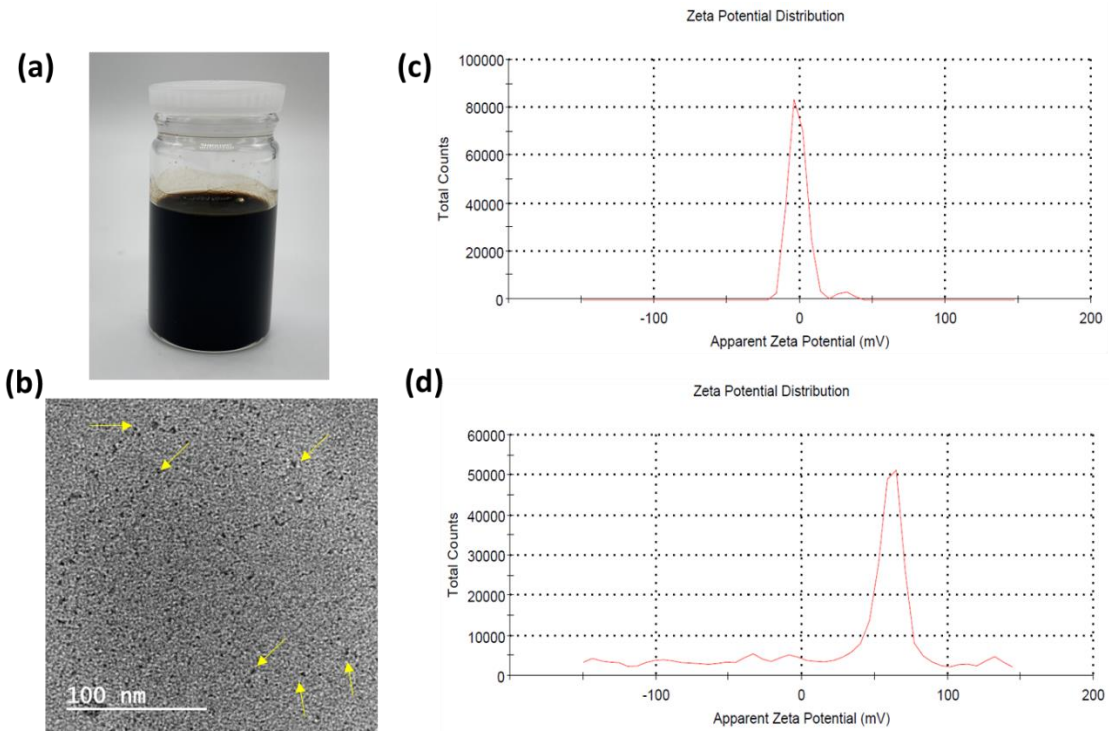

Figure 2 (a) Photograph of as-synthesized GQDs (b) TEM image of as-prepared GQDs (black dots indicated with yellow arrow) (c) Zeta potential of bare GQDs and (d) surface functionalized GQDs.

2b. Assays for siRNA delivery and effects of two or more siRNA oligos.

For the two siRNA oligos targeting *MGV1*, treatments with a combination of 10% MGV1-A and 10% MGV1-B siRNA oligos did not have stronger inhibitory effects than treatments with individual siRNA oligos (at 20%). Additional tests with siRNA oligos targeting other genes will be pursued (siRNA oligos were used up and back ordered).

2c. Determine accumulative effects of simultaneously silencing of two or more genes.

We also tested with combining MGV1-A and COT1-A siRNA oligos on conidium germination and germ tube growth in *F. graminearum*. Our preliminary data showed that combining MGV1 and COT1 siRNA oligos had no synergistic effects for these two genes.

Objective 3. Target gene prioritization, dsRNA design with cell-penetrating facilitation, and spraying and phenotyping FHB inoculated susceptible varieties by nanomaterial coated dsRNAs

3a. Prioritization of *Fg* genes, development and dsRNA design with cell-penetrating facilitation for applications on wheat.

In the project proposal, we have listed genes that were identified or recommended by co-PI Xu for targeting in wheat-Fusarium interaction research. These genes included *MGV1*, *RAS1*, *COT1*, *YCK1*, and *CAK1* genes that are important or essential for growth (Hou et al. 2002, Bluhm et al. 2007, Wang et al. 2011), *CID1* (Zhou et al., 2010), *Fgpal1* (Yin et al., 2020) *FgPp2A* & *FgPpg1* (Yu et al., 2014), and *FTL1* (Ding et al., 2009), that were all previously published by Xu laboratory. We only started designing and producing dsRNA tools targeting two genes *MGV1* and *RAS1*. To design RNAi constructs that could silence each target gene, we used the plant-specific dsRNA design pssRNAit server (Ahmed et al., 2020). A 500-700 bp fragment from the coding region was used for prediction of siRNA sites. Parameters were set to maximize the number of siRNA sites in 100-300 predicted region and minimize any off-target region. After identification of the most suitable region, we used the native stretch of DNA predicted pssRNAit to have greatest number of predicted siRNAs, and called it a “native” segment. In addition, we used each of the predicted siRNA in tandem one after another in a hypothetical manner that does not exist in nature. We named this hypothetical tandem a “synthetic” segment. We then synthesized both native and synthetic segments for each gene by commercial DNA synthesis providers. For each of the Fusarium genes *MGV1* and *RAS1* and the wheat *phytoene desaturase* (*PDS*) gene we produced a native and a synthetic segment and cloned them in the double T7 promoter vector L4440 (Addgene 1654). The recombinant L4440 was transformed into the mutant strain HT115 (DE3) by a standard transformation procedure. This strain lacks RNase III, which is a dsRNA degradation enzyme. Therefore, the dsRNA produced in this bacterium will not be degraded. The L4440 also has T7 RNA polymerase-mediated transcription promoter which is induced by isopropyl β-d-1-thiogalactopyranoside (IPTG). For induction and large-scale production of dsRNA, the procedure developed by Lisa Timmons (Carnegie Institution of Washington), with some modifications based on Zhang et al (2019) and Ahn et al., (2019) was followed. Recent production resulted in a bulk of native and synthetic dsRNA yields of 100 ng/uL and 185 ng/uL, respectively for these genes.

3b. Treating FHB inoculated susceptible varieties by nanomaterial coated dsRNA design, and performing FHB phenotyping to characterize the efficacy of non-GMO gene silencing strategy.

The first round of our dsRNA production was completed near the end of the winter. Due to hot greenhouse season, we were not able to grow and test FHB infection in the greenhouse and test the efficacy of the dsRNA crude extracts on prevention of disease. However, we were able to develop the same strategy by the use of a marker gene that is commonly used for most silencing applications in wheat. Similar to the approach taken for *MGV1* and *RAS1*, we used phytoene desaturase (PDS) and designed native and synthetic constructs. We are optimizing the treatment variations such as with or without oil bodies, spray versus infiltration to examine whether dsRNA tools designed based on coding sequence of PDS can result in bleaching of the leaves. We are also collaborating with RR group to test the efficacy of dsRNAs coated with graphene quantum dots (GQD) nanoparticles. Postdoc in RR lab and graduate student in MM lab are working on the preparation of formulation for the graphene quantum dots (GQD). Various concentrations of GQD in response to the concentrations of produced dsRNA will be tested. It will help to decipher the best combination of dsRNA with the GQDs for the phenotypic study.

What were the significant results?

Obj1. In the first phase of the project, we used chitosan for si RNA coating, which chitosan itself resulted in the arrest of fungal growth. We have found an effective alternative process to chitosan nano particles by using graphene quantum dots. The process has shown successful results and we will further continue the efforts on using this nanomaterial on plants. **Obj2**. We tested two different types of nanomaterials to coat siRNA oligoes for F. graminearum treatments. Two siRNA oligos each were tested for the *MGV1* and *COT1* genes. The effects of combining two siRNA oligos targeting the same or different genes were tested. **Obj3**. A plant-specific prediction server (pssRNAit - Ahmed et al., 2020) was used to design native and synthetic dsRNA silencing tools. Both native and synthetic tools were synthesized. Compared to the high-cost in-vitro transcription methods, we were able to source L4440 vector coupled with the HT115 mutant to produce large scale sRNA in-vivo for three genes i.e., wheat PDS and Fg *MGV1* and *RAS1*.

List key outcomes or other achievements.

Obj1. There is a high likelihood of having multiple high impact publications and potential patent on the new process that has been identified by the team, in particular the interface on nanotechnology and plant biology. **Obj2**. Graphene Quantum Dots (GQDs) were found to be more suitable than chitosan nanoparticles. Treatments with siRNA oligos targeting were found to inhibit conidium germination and germ tube growth in *F. graminearum*. Treatments with GQDs coated PMK1 siRNA oligo were inhibitory to appressorium formation in the rice blast fungus, confirming the effects of gene silencing by GQDs coated siRNA oligos. **Obj2**. The dsRNA targeting PDS marker gene has once been applied by infiltration to the detached leaves of wheat for the first observation. We need to conduct more experiments with several changes in applications treatments in order to learn more about this technique. The production of dsRNA targeting *MGV1* and *RAS1* were optimized in mutant strait HT115 by using inducible double T7 promoter that will be used on Fg-inoculated susceptible wheat plants in the fall greenhouse season.

3. What opportunities for training and professional development has the project provided?

Obj1. Devendra Sarnaik is graduate student and Sachin Kadian is post-doctoral student working on the project (RR research group). **Obj2**. A MS student, Penelope Vu, was trained in this project (JRZ research group). **Obj3**. Binod Gyawali, a graduate student has started his dissertation thesis on Obj3. He is actively involved in dsRNA design, cloning, and production of dsRNA for testing (MM research group).

4. How have the results been disseminated to communities of interest?

The team is working on finalizing the recently collected results to present at USWBSI Annual Forum and MRS conference. MM is anticipating that, if results are satisfactory, the graduate student Binod Gyawali presents a poster from his marker gene PDS silencing in the USWBSI Annual Forum.