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Editing *Fusarium graminearum* using CRISPR/Cas9

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EDITING *FUSARIUM GRAMINEARUM* USING CRISPR/CAS9

A Thesis Submitted

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Abstract

Fusarium graminearum is a pathogenic fungus that causes scab or Fusarium head blight disease in barley and wheat. Alternative approaches to protect barley from this devastating disease are badly needed. In recent years, an RNA-guided genome-editing system known as clustered regularly interspaced short palindromic repeat and CRISPR-associated protein 9 (CRISPR/Cas9), has become a popular method for genome modification (Jinek et al., 2012). We applied CRISPR/Cas9 technology in an effort to make *F. graminearum* less infectious. We used CRISPR/Cas9 plasmids constructed by a former UNI student (Akers, 2019) to mutate three genes in *F. graminearum*. One of the genes (*AUR1*) is a visual marker and the other two genes (*MGV1* and *Tri5*) are essential for infection. The CRISPR/Cas9 plasmids also contained a hygromycin B resistance gene for selection. We transformed *F. graminearum* protoplasts and selected colonies on media containing hygromycin B. We hypothesized that transforming *F. graminearum* protoplasts with CRISPR/Cas9 plasmids would edit the target genes. We recovered many colonies on selective media. This suggests that colonies carry the CRISPR/Cas9 plasmids. However, we were unable to produce data that shows mutation in the genome, partly because of the shutdown of campus due to the COVID-19 pandemic.

Acknowledgements

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Grace Sack

Advisor: Dr. Tilahun Abebe

Table of Contents

Introduction

Fusarium graminearum is a pathogenic fungus that causes scab or *Fusarium* head blight disease in barley and wheat. The disease not only reduces yield, but it also contaminates the kernel with harmful toxins. Therefore, the disease causes economic loss and poses a health risk to humans and animals. Previous studies have shown that CRISPR/Cas9 can be used to introduce mutations in filamentous fungi (Nødvig et al., 2015). In this study, we used CRISPR/Cas9 to mutagenize three genes in the filamentous fungus, *F. graminearum*: *MGV1* (essential for sexual reproduction and infection), *Tri5* (important for toxin production and infection) and *AUR1* (a visual marker). *F. graminearum* is a pathogenic fungus that infects cereal crops such as barley and wheat. We hypothesize that silencing *MGV1* and *Tri5* could inhibit the ability of the fungus to infect these crops. We targeted *AUR1* for silencing, because this gene is an effective visual marker for mutagenesis.

Purpose

The purpose of this research project is to silence three genes (*Tri5*, *MGV1*, and *AUR1*) in the pathogenic fungus *Fusarium graminearum* using CRISPR/Cas9.

Literature Review

Barley and wheat are cereal crops that are of tremendous importance to the United States agricultural industry. These crops are a critical food source for people and livestock, and they provide a significant economic contribution due to their role as a major U.S. export. Based on production tonnage, wheat and barley are the second and fourth largest cereal crops produced in the world, respectively (FAS, 2019). Wheat is the second largest cereal crop produced in the U.S., and barley is the fifth largest (NASS, 2019).

Figure 1. Infected (left) vs. healthy barley (right). Source: USDA

Scab or *Fusarium* head blight is a devastating disease of wheat and barley (Tekauz et al., 2000, Rubella et al., 2004). The disease is caused by the pathogenic fungus Fusarium graminearum (Osborne and Stein, 2007), which infects the kernel (Figure 1). In barley and wheat, infected heads have sterile florets, shriveled and discolored kernels, reduced grain weight and yield. Scab also contaminates the grain with harmful toxins, primarily deoxynivalenol (DON). Therefore, the disease poses a health risk to humans and animals. The USDA has set guidelines for maximum acceptable levels of this toxin (5 ppm), but even greater restrictions have been self-imposed by many food and beverage industries (McMullen et al., 2012).

F. graminearum reproduces both sexually (meiotic division) and asexually (mitotic division, Figure 2). Spores produced through sexual reproduction are known as ascospores and those produced by asexual reproduction are called macroconidia. Ascospores are produced in a specialized multicellular structure called perithecium (fruiting body). Perithecia form on infected

plant debris. Ascospores are essential for overwintering and are the primary inoculum (Trail et al., 2002; Guenther and Trail, 2005). In the spring, ascospores settle on the head and infect the seed (Rubela, et al., 2004). Thereafter, asexual spores (macroconidia) are produced in conidiophores on infected crops in a cushion-shaped hyphal mass called sporodochium. Macroconidia are required for secondary infection and they are the primary means for the spread of the disease.

Currently there is no natural resistance in barley, and no effective fungicides exist to protect crops either. Alternative solutions to reduce the effects of scab disease are needed (McMullen et al., 2012). In recent years, an RNA-guided genome-editing system called clustered regularly interspaced short palindromic repeats and CRISPR-associated protein9 (CRISPR/Cas9) has become a popular method for improving disease resistance in crops through genome modification (Oliva et al., 2019). This technology is a modification of the defense mechanism that many bacteria and archaea use to protect themselves against invading DNA viruses (Jinek et al., 2012).

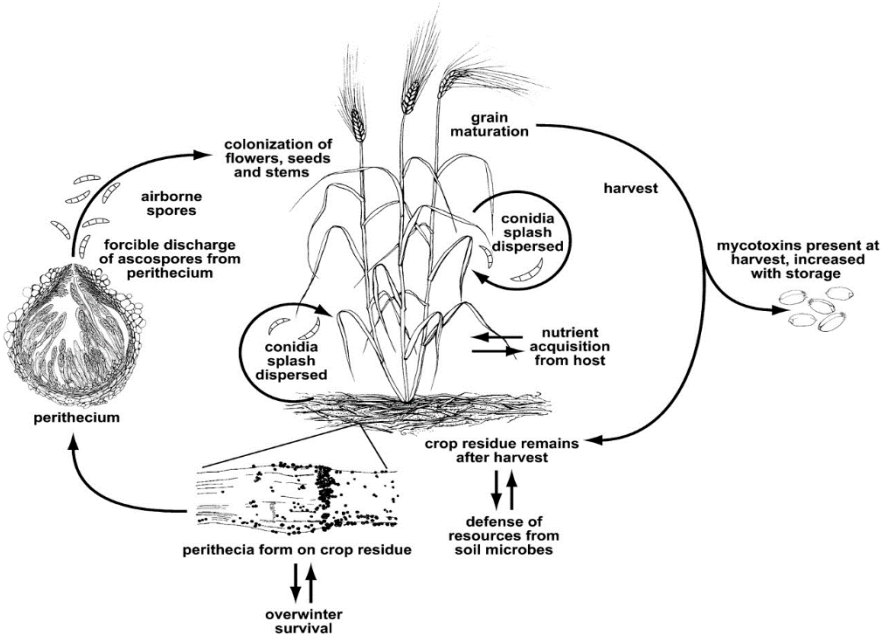

Figure 2. The life cycle of *F. graminearum* (Trail, 2009).

Previous studies have shown that CRISPR/Cas9 can be used to introduce mutations in filamentous fungi. Researchers developed CRISPR/Cas9 vectors to silence genes in *Aspergillus* species (Nødvig et al., 2015). Therefore, this technology could be adapted to silence genes in *F. graminearum* and slow its ability to infect barley and wheat. We selected three target genes in *F.*

graminearum for this research project. *Tri5* is a gene essential in production of trichothecenes, which are toxic fungal metabolites involved in infection. Trichothecenes have many effects on eukaryotic cells including inhibition of protein, DNA and RNA synthesis, inhibition of mitochondrial function, and inhibition of cell division and membrane functions. Trichothecenes also have many negative effects on the animals that ingest them, including growth retardation, reduced ovarian function and reproductive disorders, immuno-compromization, feed refusal and vomiting (Rocha et al., 2005). In wheat, the presence of trichothecenes prevents a defense mechanism that causes thickening of cell walls which can block the fungus. *F. graminearum* mutants that are unable to produce trichothecenes have been shown to produce less severe disease in wheat and winter rye (Jansen et al., 2005). Due to its role in infection and toxin production, silencing the *Tri5* gene in *F. graminearum* would make the fungus incapable of infecting its host plants.

MGV1 is important in the production of a strong cell wall, sexual reproduction, and plant infection. Mutants of *F. graminearum* without a functional *MG1* gene produce mycelia with weak cell walls that are hypersensitive to digestive enzymes. The mutation also affects female sexual reproduction, and causes a reduction in its ability to accumulate trichothecenes on the infected plants (Hou et al., 2002). We chose *MGV1* as the second target for gene editing due to these important processes the gene is involved in.

The third gene we targeted in this project is *AUR1*. *AUR1* is required for development of the red color in *F. graminearum*. It is involved in the production of aurofusarin, a red pigment present in the cell wall (Gaffoor et al., 2005). Therefore, this gene can serve as an effective visual marker. Silencing *AUR1* produces white mycelia rather than the wild type red color. We will use *AUR1* to check whether our CRISPR/Cas9-mediated gene editing is working.

Methodology

1. Growing *Fusarium graminearum*

F. graminearum was grown on complete medium (CM) for 5-7 days at 28°C. About 2 ml of sterile water/Tween 20 was added to the mycelia. The mycelia were stirred using a bent glass rod, and the liquid was removed and added to a microcentrifuge tube. Using a hemocytometer, we calculated the density of the spore suspension. We prepared several tubes of spore suspension. The spores were dried and stored at 4°C until needed. The spores were resuspended in liquid CM to about 1.8×10^9 cells/ml. One µl of this spore suspension was added to a sterile culture flask containing 50 ml of complete medium. The spores were incubated for two days at 25 °C with shaking (160 RPM).

The mycelia were collected and homogenized using a blender. Then, 200 ml of liquid CM in a sterile culture flask was inoculated with 10 ml of this hyphal suspension. This was grown overnight in a 24°C incubator with gentle agitation (55 rpm).

2. Isolation of Protoplasts

The CRISPR/Cas9 plasmid cannot be taken in by the fungal cells if the cell wall is present. Therefore, it is necessary to isolate the protoplasts by digesting the cell wall. About 2 grams of mycelia was filtered from the overnight culture using 150 mm Whatman filter paper and added to the enzyme solution. The enzyme solution contained 0.2 g driselase and 0.5 g lysing enzyme in 20 ml of 700 mM NaCl, pH 5.6 in a centrifuge tube. The mycelia was digested for 3 hours at 28°C with gentle agitation (55 rpm).

3. Transformation of Protoplasts

Undigested hyphal material was removed by filtering the liquid through gauze. The gauze was rinsed with 20 ml of 700 mM NaCl, pH 6.5. Protoplasts were pelleted by centrifugation at

1,300× g in a swing-out rotor for 3 min. The pellet was washed twice in 10 ml ice-cold 700 mM NaCl, pH 5.6 each and centrifuge at 830× g for 3 min. We resuspended protoplasts in 150 µl STC (50 mM Tris-HCl, pH 8.0; 0.8 M sorbitol; 50 mM $CaCl_2$) and determined the density using a hemocytometer. We routinely get 10^9 protoplasts.

To mutate the three genes in *F. graminearum*, we used CRISPR/Cas9 plasmids constructed by a former UNI student (Akers, 2019). These plasmids are based on the vectors by Nødvig et al. (2015). The basic structure of the CRISPR/Cas9 plasmids is shown in Figure 3. One hundred µl of the protoplast suspension was added to three 1.5 ml tubes. Five µl of heparin was added to each tube as an anticoagulant. Then, 10 µl of CRISPR/Cas9 plasmid was added to the corresponding tube for each of the three target genes. The tubes were incubated on ice for 30 minutes. One ml of SPTC (40% polyethylene glycol 4000 in STC) was added to each tube, and then the tubes were incubated at room temperature for 20 minutes. We transferred each transformation reaction to a 50 ml tube containing 45 ml of regeneration medium kept at about 50°C. Each tube was mixed and plated on five 100×15 ml plates (15 plates total). The plates were incubated overnight at 28°C. The next day, the plates were overlaid with selective agar that contained 1.2% agar and 300 mg/L the antibiotic hygromycin B.

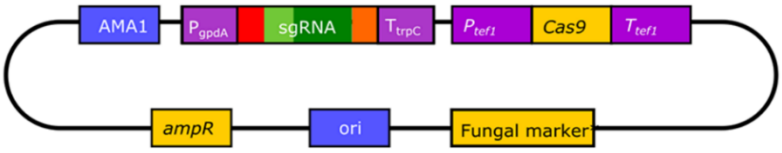

Figure 3: Basic plan of the CRISPR/Cas9 plasmids. The single guide RNA (sgRNA) is shown in light green (Nødvig et al., 2015).

4. Selection of Transformed *F. graminearum* Colonies

Only transformed colonies are expected to grow on media containing hygromycin B. After several days of incubation, colonies growing on selective agar plates were transferred to

fresh CM plates containing 100 mg/L hygromycin B. These colonies were screened by PCR to determine mutation of the target genes.

5. Screening Colonies for Mutations

We screened colonies for transformation as well as mutation using the polymerase chain reaction (PCR). We performed a multiplex PCR using two primer pairs: One set was used to amplify a portion of the plasmid DNA introduced into the fungus (CSN389 and CSN390). This would tell us whether we succeeded transforming *F. gramine*arum by the CRISPR/Cas9 plasmids. Another set of primers was used to amplify a segment of the gene targeted by CRISPR/Cas9 (AUR1 forward, AUR1 reverse; MGV1 forward, MGV1 reverse, and Tri5 forward, Tri5 reverse; Table 1). A reduction in the size of the amplified regions compared with the wild type would indicate that the target genes were cut by the Cas9 enzyme. We separated the PCR products on 1.2% agarose gel electrophoresis. Components of the PCR and the PCR conditions are presented in Tables 1 and 2.

Table 1. Primers used to detect CRISPR/Cas9 mediated mutations in *F. graminearum*.

Primer pair	**Sequence**	**Target gene**	**Size of intact fragment**
AUR1_For219 AUR1_Rev891	5′-AGCCACTCAAGTATGCAGAT-3′ 5′-TCTCTCCAATCATCGCTAGT-3′	AUR1	673 bp
MGV1_For375 MGV1_Rev1161	5′-TGTTTGGTCTGTTGGTTGTA-3′ 5′-TTTCCATCCTTCTCTTCTCA-3′	MGV1	787 bp
Tri5_For191 Tri5_Rev706	5′-AGGCTTCCCTCCAAACAATC-3′ 5′-CAAACCATCCAGTTCTCCATCT-3′	Tri5	536 bp

Table 2. Components of the polymerase chain reaction

Component	Volume for 1 reaction
Sterile water	4.5 µl
2× GoTaq Mix	12.5 µ
MGV1 or AUR1 or Tri5 forward primer (5 µM)	1 µl
MGV1 or AUR1 or Tri 5 reverse primer (5 µM)	1 µl
CSN444 primer	1 µl
CSN390 primer	1 µl
DNA	4 µl
Total volume	25 µl

Table 3. PCR conditions.

Cycle	Temperature (°C)	Time
Initial Denaturation	98.0	30:00 min
Denaturation	94.0	10 sec x 35
Annealing	65.0	5:00 x 35
Extension	72.0	1:00 x 35
Hold	15.0	Hold

Results

1. Cell Wall Digestion

We observed the appearance of the *F. graminearum* before and after enzyme digestion to verify that the cell wall had been degraded. We found that driselase is the only enzyme that can degrade the cell wall of *F. graminearum* (Figure 3). After two transformation attempts, we ran out of driselase and the supplier, Millipor Sigma, has discontinued this enzyme. We attempted to

digest the cell wall using Vinotaste and higher concentrations of the lysing enzyme, but none of these enzymes were able to digest the cell wall of *F. graminearum* separately or in combination.

(a) (b) (c)

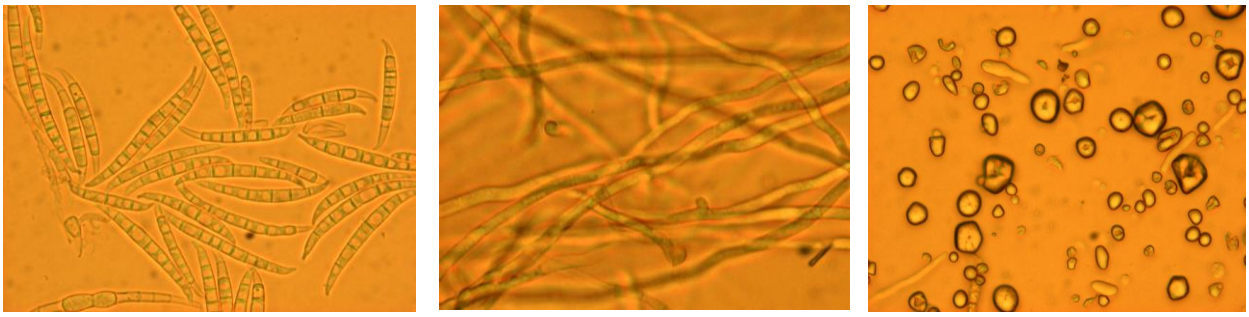

Figure 4. Germination of *F. graminearum* spores and isolation of protoplasts. (a) Macroconidia, (b) mycellia two days after germination of spores, and (c) protoplasts after enzyme digestion

2. Transformation of *F. graminearum* protoplasts

Fungal colonies appeared on selective agar plates after several days of incubation (Figure 5). Then, we cut out these colonies, along with the surrounding medium, using a wide-bore pipette tip and a toothpick. The agar plugs were transferred to new CM plates containing 100 mg/L hygromycin B. Mycelia that grew on fresh media were transferred to another CM plate with hygromycin B for purification of colonies (Figure 6).

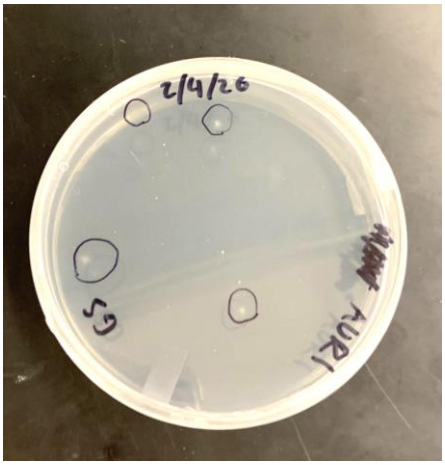

Figure 5. *F. graminearum* colonies on selection medium.

The resulting colonies exhibited morphology that appeared different from the wild type *F. graminearum* we started with. The fungi did not exhibit the fuzzy, white mycelium found in wild type *F. graminearum.*

(a) (b) (c)

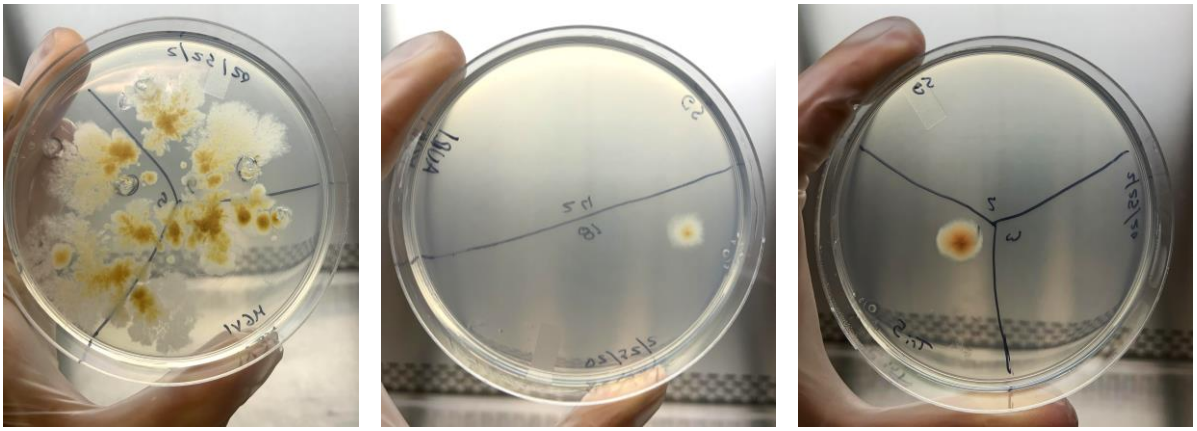

Figure 6. Colonies after transfer to another selection plate. (a) MGV1 (b) AUR1, (c) Tri5.

3. Detection of CRISPR-Cas9 Mediated Mutation

We expected two bands in the fungal samples, one representing the target genes and the other derived from the plasmid. The wild type lane showed no bands. Most of the fungal colonies produced one band smaller than 915 base pairs. One fungal colony (#10) showed a second band greater than 915 base pairs. The negative control was expected to be blank, but it showed one band, indicating a mistake had been made during the PCR procedure (Figure 7).

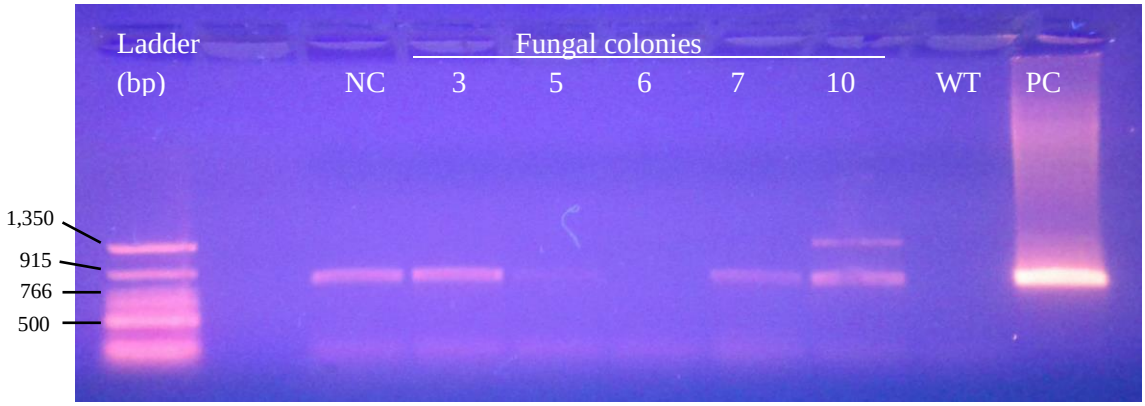

Figure 7. PCR amplification of *MGV1* region flanking the Cas9 target. Ladder, Quick-Load® 50 bp DNA Ladder (NEB, Catalog # N0473), NC indicates negative control, PC indicates positive control, and WT indicates wild type *Fusarium graminearum*.

Discussion

Microscopic evaluation of *F. graminearum* mycelia supports that we successfully digested the cell wall with Driselase/lysing enzyme and isolated protoplasts (Figure 3). This was necessary to introduce the CRISPR/Cas9 plasmids and to edit the target genes.

After the transformation procedure, *F. graminearum* samples were recovered on selective media containing the antibiotic hygromycin B. Only transformed colonies that express the hygromycin resistance gene should be able to grow on this media, which suggests transformation occurred. The mycelia were purified and allowed to grow into larger colonies. These colonies appeared to have morphological differences from the *F. graminearum* we started with. We believe this could also suggest that genetic silencing occurred. *MGV1* is a gene essential for cell wall integrity. Mycelia with edited *MGV1* lacked the ability to grow a mass of mycelia upwards like those from the wild type *F. graminearum*. The mycelia grew flat and shiny rather than having the wild type fuzzy appearance. Only one *AUR1* colony was produced. It had a yellowish-white appearance, rather than having the typical red and white color. Several *Tri5* colonies were recovered. These colonies had a reddish-yellow appearance, and also did not have the appearance of wild type *F. graminearum*.

We attempted to use polymerase chain reaction (PCR) to produce data that supports that genetic editing had occurred. By using two sets of primers, we expected to see two bands in the fungal colonies that were recovered. Differences in size would have indicated that the target genes were edited, since we know that *MGV1* is 787 bp, *AUR1* is 673 bp, and *Tri5* is 715 bp. Most of the colonies only produced one band, and each appeared to be the same size as the plasmid DNA (positive control) band, which could indicate plasmid DNA was present in the fungal colonies. Only one colony produced two bands. This could indicate the genome had been

mutated. However, the negative control produced one band, which should have been blank. It is likely that tubes were switched, since two other lanes did not show any bands, including the wild type control. This supports that the master mix was not contaminated. Because of these confusing results, the PCR should have been repeated with these fungal colony samples. Unfortunately, we were not able to continue the research due to the COVID-19 crisis. In the future, students will perform new PCR using the fungal samples from the most recent transformation procedure.

Conclusion

F. graminearum is a pathogenic fungus that causes adverse economic and health effects. We hoped to decrease the ability of this fungus to infect cereal crops with the application of CRISPR/Cas9 technology. We anticipated that transforming *F. graminearum* with CRISPR/Cas9 plasmids would silence the genes *Tri5*, *MGV1*, and *AUR1* and reduce *F. graminearum's* ability to infect barley. We were unable to confirm whether we transformed *F. graminearum* and edited the target genes. Further research is needed to screen the *F. graminearum* colonies we produced for mutations. Sequencing the target genes would be a better approach than gel electrophoresis since 1.2% agarose gel would not be able to detect if the deletion is smaller than 50 - 100 bases.

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