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Construction of CRISPR/Cas9 vectors for directed mutagenesis of Fusarium graminearum

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CONSTRUCTION OF CRISPR/CAS9 VECTORS FOR DIRECTED MUTAGENESIS OF *FUSARIUM GRAMINEARUM*

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Date Dr. Tilahun Abebe, Honors Thesis Advisor, Department of Biology

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Date Dr. Jessica Moon, Director, University Honors Program

Abstract

Fusarium graminearum causes the fungal disease known as scab or head blight in cereals, including barley. There is no known resistance in barley against scab disease. Generating mutations in the fungus is a promising strategy to reduce infection. Research has shown that CRISPR/Cas9 is a very powerful tool for gene mutation. In this study, CRISPR/Cas9 vectors containing guide RNA sequences were constructed to mutate one visual marker gene (*AUR1*) and two genes essential for infection in *F. graminearum*(*Tri5* and *MGV1*). Plasmids pFC332 and pFC334 were used to create vectors with the target genes using the USER cloning kit. The vectors were cloned into *E. coli* sand confirmed the presence of guide RNA sequences and accompanying sequences (promoter, terminator, and tracrRNA) using PCR and sequencing. The vectors will be used to mutagenize *AUR2, Tri5*, and *MGV1* genes in *F. graminearum* in future studies. It is predicted that mutated *F. graminearum* would not be able to infect host barley (*Hordeum vulgare*) plants.

Acknowledgments

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Introduction

Fusarium head blight is a devastating cereal disease caused by the pathogenic fungus, *Fusarium graminearum* (Osborne and Stein, 2007). *Fusarium* head blight, also called scab disease, causes tremendous yield losses in many cereals including barley, wheat, and corn (Tekauz *et al*., 2000, Rubella *et al.*, 2004). There is no effective natural resistance in barley against *F. graminearum*. However, the rapid availability of the whole genome sequence of *F. graminearum* (Cuomo *et al*., 2007; King *et al.*, 2015) has opened a new chapter in the search to find a solution against *Fusarium* head blight. The sequenced *F. graminearum* genome is critical to advance the research aimed at understanding the pathogenic process including penetration, host tissue necrosis, and host immune subversion.

Gene silencing strategies using RNA interference (RNAi) have shown some promise in enhancing resistance against this pathogen (Kosh et al., 2016). In the past few years, clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (Cas9) has become a powerful alternative to mutate target genes in any eukaryote (Jinek et al. 2012). In this study, new vectors were created to edit selected genes in *F. graminearum*. The vectors developed will be used to mutagenize *F. graminearum* in future research. In a future study barley plants will be infected with mutant *F. graminearum* to assess the effectiveness of this approach in reducing infection. Furthermore, new vectors carrying the guide RNA sequences targeting the genes in *F. graminearum* will be introduced into barley and the transgenic plants will be infected with the pathogen to test if this approach eliminates the threat. Genetically modified organisms are becoming more common and modifying traits is presenting more options to improve resistance and increase crop production.

Purpose

There are no resistant barley cultivars against the fungal pathogen *F. graminearum*. The purpose of this project was to mutate three genes (*AUR1, Tri5* and *MGV1*, and) in *F. graminearum*. *AUR1* is involved in the biosynthesis of the red pigment aurofusarin in *F. graminearum*, primarily the perithecia (Gaffoor *et al.*, 2005), which is a good visual marker of genetic disruption. *Tri5* and *MGV1* are essential for the fungus to infect its host. It is anticipated that mutating these genes will make the fungus less infectious. Development of transgenic crops overexpressing antifungal genes increases production of healthy barley across North America.

Literature Review

F. graminearum is the most common cause of *Fusarium* head blight in North America and many other temperate and subtropical parts of the world including Europe, South America, and Asia. The pathogen infects the kernel and has the capability to destroy a potential high-yielding crop within a few weeks. In barley and wheat, infected heads have sterile florets and produce shriveled kernels with light test-weight (Figure 1). *F. graminearum* reproduces both sexually (meiotic division) and asexually (mitotic division) depending on environmental conditions (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), which forms on infected plant debris. Ascospores are essential for

Figure 1. Infected versus healthy head (https://www.countygp.ab.ca/EN/main/departments/agriculture/pest-disease-control/barley-fusarium-head-blight.html, accessed 26 October 2018.)

overwintering and are the primary inoculum (Trail *et al*., 2002; Guenther and Trail, 2005). In the spring, ascospores settle on the spike (head) and infect the seed (Rubela, *et al.*, 2004). Thereafter, the 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 *F. graminearum* in the field (Guenther and Trail, 2005; Desjardin *et al*. 2006).

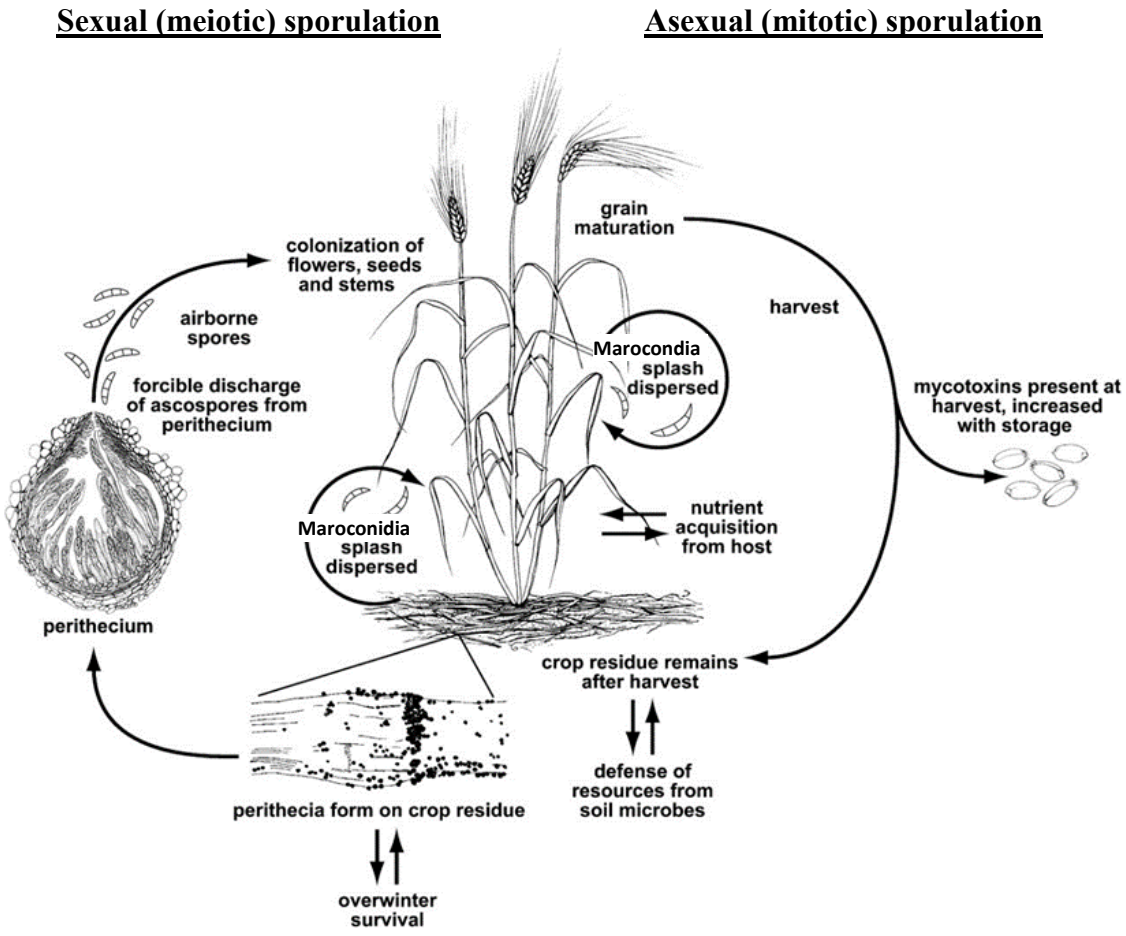

Figure 2. The life cycle of *F. graminearum* (Trail, 2009)

Besides substantial yield loss, the quality of infected kernels is compromised. Kernels infected with *F. graminearum* become discolored and accumulate mycotoxins, primarily deoxynivalenol (DON). Consumption of kernels contaminated with mycotoxins is harmful to humans and animals, leading to growth retardation, reproductive disorders, immunosuppression, neurotoxicity, and altered nutrient uptake (Hsia *et al*., 2004). In domestic animals, particularly swine, DON induces vomiting and protracted feed refusal.

Development of transgenic crops overexpressing antifungal genes are promising in response to this pathogenic fungus (Kazan *et al*., 2012; Koch *et al*., 2013). CRISPR-associated protein 9 (Cas9), could become an alternative for precise sequence-specific genome modification (Jinek *et al*., 2012). CRISPR/Cas is a defense mechanism that many bacteria and archaea deploy against invading DNA viruses and plasmids. The system uses short guide RNAs to target the Cas nuclease to cleave the invading DNA on specific regions. The system is so powerful that many researchers have engineered it to mutagenize eukaryote genomes, including mammalian as well as plant genomes.

A typical bacterial CRISPR locus (region) from *Streptococcus pyogenes* consists of a repeat spacer-array (known as CRISPR array), CRISPR-associated (cas) genes, and a trans-activating RNA (tracrRNA) region (Figure 3). The CRISPR array is composed of a series of short sequences (protospacers) derived from invading DNA viruses or plasmids separated by small palindromic repetitive DNA sequences (repeats). The CRISPR array is transcribed into a crRNA precursor (pre-crRNA). The RNA transcribed from the tracrRNA region

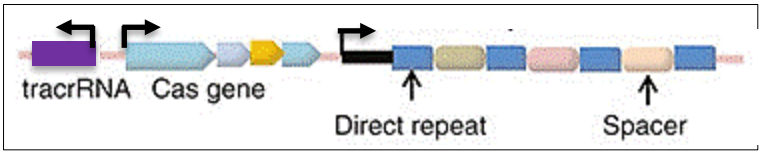

Figure 3. Processing of crRNA. Modified from Alkhnbashi *et al*. (2014).

has two segments, a portion of the tracrRNA binds to the Cas9 protein and the other segment is hybridized to the repeats in the pre-crRNA. The pre-crRNA, several tracrRNAs and Cas9 proteins form a complex. This complex is processed by RNAse III enzymes (biogenesis of crRNA) into several effector complexes, each containing a crRNA, a tracrRNA, and a Cas9 protein). The first time the virus attacks, the bacterium shears the viral genome using the cas proteins and incorporates a short fragment of the invading DNA at the 5′ end of the CRISPR array (acquisition phase). The CRISPR array with the new protospacer is transcribed into a pre-crRNA and processed into an effector complex consisting of a tracrRNA, a crRNA, and a Cas9 protein (processing phase). The next time the virus attacks, the CRISPR/Cas effector complex uses the protospacer to identify and destroy the intruder (interference or immunity phase) (Figure 4).

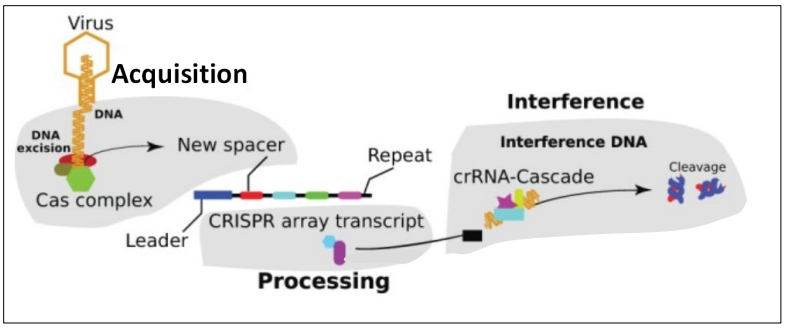

Figure 4. The type II CRISPR/Cas9 system in *Streptococcus pyogenes*. Modified from Dai *et al*. 2016. Mol Ther Nucleic Acids 5:e349. doi: 10.1038/mtna.2016.58.

In nature, the pre-CRISPR RNA (pre-crRNA) and the trans-activating crRNA (tracrRNA) are transcribed as separate RNA molecules. In the CRISPR/Cas9 system, engineered for editing target genomes, the tracrRNA and crRNA are connected by a short linker sequence to form a single guide RNA (sgRNA) (Figure 5). This single RNA chimera directs Cas9 for sequence-specific cleavage in target DNA as the natural one. Accurate targeting of the RNA-guided Cas9 nuclease to a specific DNA sequence is achieved by introducing a short (20 nucleotides long) protospacer sequence in the sgRNA. The sgRNA guides Cas9 to the precise spot on the target DNA, creating a break in both strands of the DNA molecule. RNA guided

DNA double strand breaks generated by Cas9 are repaired through a process known as non-homologous end joining (NHEJ). NHEJ is error-prone and leads to deletion of one to hundreds of nucleotides in the target region which can lead to complete inactivity of a gene. The only restriction of the CRISPR/Cas9 system is the requirement for a short (three nucleotides) protospacer adjacent motif, PAM, in the target DNA immediately after the binding site of the guide RNA. The target site is cleaved 3-5 bp upstream of the PAM site.

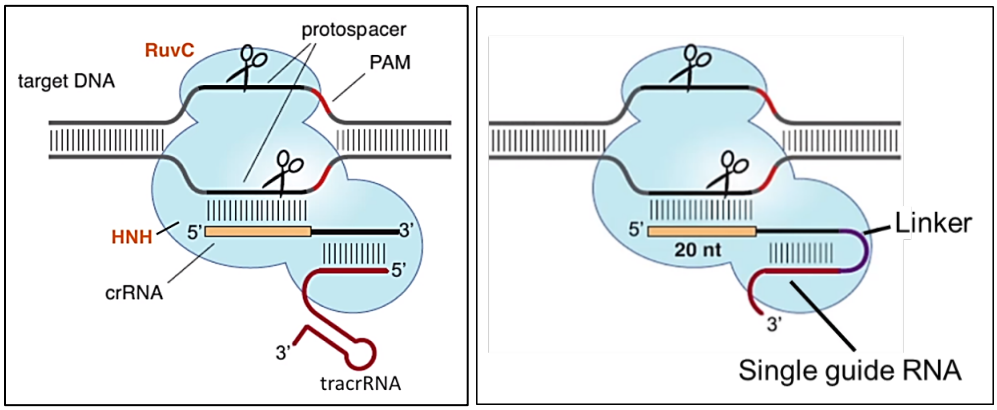

Figure 5. Schematics of the *S. pyogenes* CRISPR/Cas9 system. (Top) The Cas9 protein consists of two unrelated domains: a nuclease (NUC) lobe and a recognition (REC) lobe. The REC domain cuts the target DNA strand matching the protospacer and the REC domain cuts the nonmatching strand. (Bottom) In the engineered CRSPR/Cas9, the crRNA and tracrRNA sequences are connected by a short linker DNA to create a single chimeric guide RNA (sgRNA). The sgRNA still interacts with and guides Cas9 to the target DNA. This bypasses the requirement for RNase III. (Jinek, *et al.*, 2012).

Mutations using CRISPR/Cas9 can be introduced in three ways: (i) by transforming target cells with a plasmid carrying DNA sequences for the guide RNA and the Cas9 enzyme, (ii) by injecting the guide RNA and the Cas9 mRNA into the target cell or (iii) by injecting the guide RNA and the Cas9 enzyme into the target cell. Choice of these methods depends on the cells used since not all cells are amenable to all approaches. In the work presented here, we constructed CRISPR/Cas9 plasmids to mutate three genes (described later) in *F. graminearum* using the first approach.

Target Genes for Mutation in *F. Graminearum*

Transformation-mediated gene disruption can lead to easily detectable disruption of target genes. In the study presented here, I used CRISPR/Cas9 to disrupt three genes (*AUR1, Tri5*, and *MGV1*) in *F. graminearum*. *Tri5* and *MGV1* are essential for *F. graminearum* to infect its host. By disrupting these genes, it is hypothesized infection would be less detrimental to the host plant, barley.

The red pigmentation of *Fusarium graminearum* and related species that cause stem and head blight of cereals is due to the deposition of the carmine pigment aurofusarin in the cell walls. Disruption of aurofusarin biosynthesis produces an albino phenotype. However, the color change due to inhibition of aurofusarin does not appear to aid in radiation protection and all the mutants are fully pathogenic on wheat and barley (Malz *et al.*, 2005). Multidomain enzymes classified as type I polyketide synthases (PKSs) are responsible for aurofusarin biosynthesis. Based on the striking phenotype of the disrupted mutants, *AUR1* was identified as the key PKS for the biosynthesis of aurofusarin (Gaffoor *et al.*, 2005) and is an effective visual marker of genetic disruption. We included *AUR1* to test the feasibility of using CRISPR/Cas9 in *F. graminearum*. Mutation of this gene is easy to monitor, Inactive AUR1 is produces white conidia (asexual spores) rather than the green color characteristic for wild-type conidia spores.

Trichothecenes are one of the major *Fusarium* mycotoxins synthesized mainly by the members in the *Fusarium graminearum* species complex (FGSC). The fungi in the FGSC have the potential to devastate a crop by reducing grain quality and quantity. The *Tri5* gene codes for a trichodiene synthase enzyme that begins the DON (deoxynivalenol, a trichothecene mycotoxin) biosynthesis pathway by the cyclization of the initial substrate, farnesyl pyrophosphate (FPP) to produce non-toxic trichodiene. The next nine reactions in the pathway promote the spread of *F.*

graminearum to infection within the host plant. The *Tri5* gene is strongly expressed in the main stem tissue of wheat (Amarasinghe CC & Fernando WGD, 2016).

Trichothecenes are important for *F. graminearum* to infect the cereal head. In the absence of trichothecenes, the fungus is blocked by the heavy cell wall thickenings in the rachis node of wheat. This defense is inhibited by the mycotoxin. In barley, hyphae of both wild type and a trichothecene mutant, are inhibited at the rachis node and rachilla, limiting infection of adjacent florets through the phloem and along the surface of the rachis. Mutant strains incapable of producing trichothecenes because of disruption of the trichodiene synthase gene *Tri5* exhibit reduced severity of disease on wheat and winter rye in field trials (Jansen *et al.*, 2005, Rocha *et al.*, 2005).

The *MGV1* gene is replaceable for conidiation in *F. graminearum* but essential for female fertility during sexual reproduction. Hou Z et al (2002) found that mutating *MGV1* in *F. graminearum*, greatly reduces accumulation of trichothecene mycotoxins on inoculated wheat. Mycelia of the *MGV1* mutants had weak cell walls and were hypersensitive to cell wall degrading enzymes. MGV1 in *F. graminearum* is involved in multiple developmental processes related to sexual reproduction, plant infection, and cell wall integrity and disease severity (Hou Z. *et al.*, 2002). Disruption of the gene is thought to be detrimental to these natural and essential characteristics of the fungus.

USER Cloning

The uracil-specific excision reagent (USER) cloning combines multiple PCR fragments, nucleotide sequence alteration, and directional cloning (Bitinaite *et al*., 2007; Vaisvila and Bitinaite, 2013). The technique involves four distinct processes: synthesizing building blocks of up to 500 bp, assembly of genes up to 3 kb, reassembly of desired sequences, and an optional

ligation into a plasmid of up to 15 kb. USER enzyme generates a single nucleotide gap at the location of uracil. The USER Cloning kit, available from New England Biolabs (catalog #M5505S), contains a mixture of uracil DNA glycosylase (UDG) and the DNA glycosylase-lyase endonuclease VIII. UDG catalyzes the excision of an uracil base, leaving the phosphodiester backbone intact. The lyase activity of Endonuclease VIII breaks the backbone at the 3′ and 5′ sides of the abasic site so that a base-free deoxyribose is released. USER Cloning results in the production of pairwise complementary overhangs at the ends of all fragments allowing selected ends to be fused in a directional manner and the PCR reagents and desired sequences to be formulated into a single, mutated plasmid (Vaisvila, R and Bitinaite, J, 2013).

Methodology

1. Synthesis of the Protospacer Sequence

CRISPR/Cas9 plasmids pFC332 and pFC334 were used to prepare plasmids to mutagenize *F. graminearum* (Figure 6). These plasmids were developed by Nødvig *et al*. (2015) to create mutations in filamentous fungi using CRISPR/Cas9. Plasmids pFC332 and pFC334 were obtained from Addgene (plasmid numbers 87845 and 87846, respectively). To express sgRNAs containing protospacers complementary to the *F. graminearum AUR1, Tri5* and *MGV1* genes, we amplified the *Aspergillus nidulans gdpA* promoter (*PgdpA*) and the *tracrRNA, HDV* and the *A. nidulans trpC* terminator (*Trpc*) region from pFC334. The *PgdpA* region was amplified using the forward primer CSN389 and reverse primer CSN444. The region containing *tracrRNA, HDV* and *Trpc* was amplified using the forward primer CSN445 and reverse primer CSN390. Primer CSN445 includes AGTAAGCUCGTC (which overlaps the 5′ end of primer CSN444) followed by 20 nucleotides of protospacer sequence, which is complementary to *Tri5*,

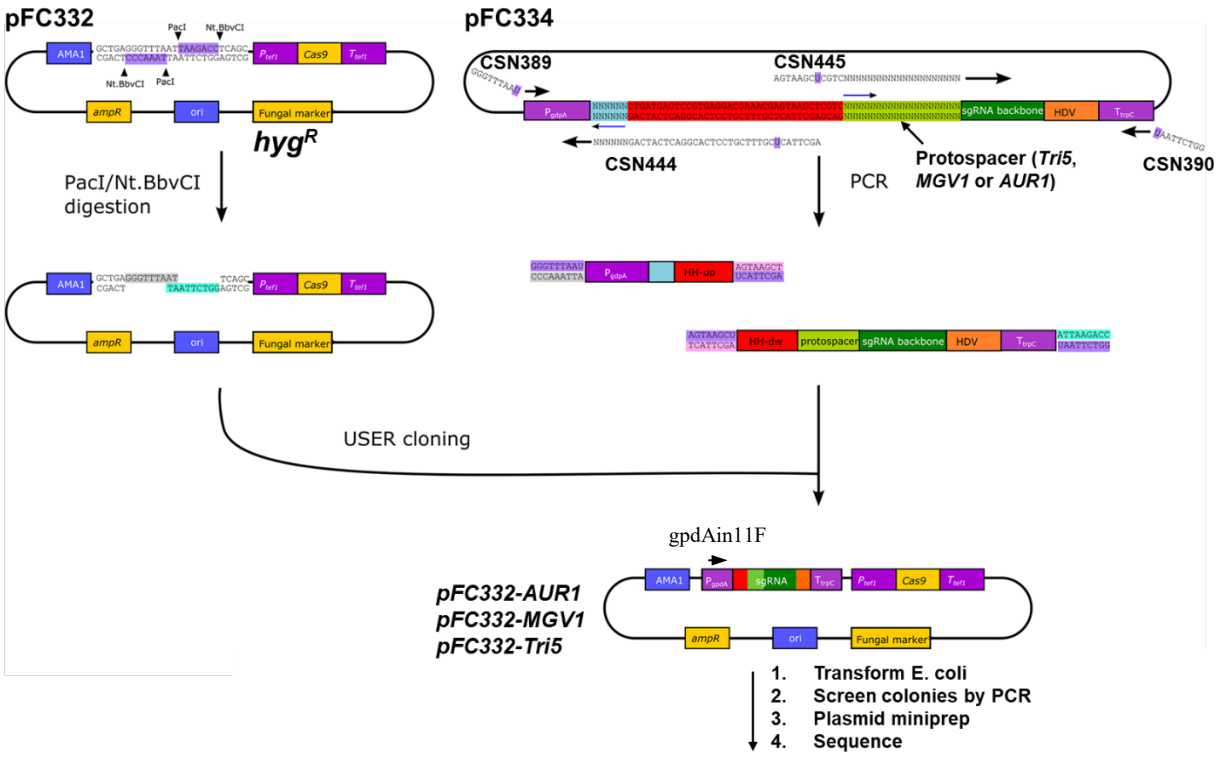

Figure 6. Flow diagram of the construction of CRISPR/Cas9 vectors for mutagenesis of *F. graminearum*. The basic plan of the three plasmids is similar except the 20 nucleotides long protospacers that are complementary to *Tri5*, *MGV1*, or *AUR1* genes in *F. graminearum*.

MGV1 or *AUR1* (Figure 6). All the primers used in this study were synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, IA). The PCR reaction contained 1 µl of 20 ng/µl pFC334, 12.5µl 2×GoTaq master mix, 1µl of each of the corresponding primers (5 µM), and 9.5 µl distilled water (Table 1). The size of the two PCR products was verified using agarose gel electrophoresis. PCR amplification was performed using GoTaq DNA master mix (Promega, Madison, WI) in MyCycler Thermal Cycler instrument (Bio-Rad, Hercules, CA) (Table 2).

PCR Component		Tube 1	Tube 2	Tube 3	Tube 4
Forward Primer	CSN389	1 µl	-	-	-
	CSN390	-	1 µl	1 µl	1 µl
Reverse Primer (5 µM)	CSN444	1 µl	-	-	-
	CSN445-Tri5	-	1 µl	-	-
	CSN445-MGV1	-	-	1 µl	-
	CSN445-AUR1	-	-	-	1 µl
2×GoTaq Mix	12.5 µl	12.5 µl	12.5 µl	12.5 µl	12.5 µl
pFC334 (20 ng/µl)	1µl	1µl	1µl	1µl	1µl
Water	9.5 µl	9.5 µl	9.5 µl	9.5 µl	9.5 µl

Table 1. Components used in the PCR

	Temperature (°C)	Time
Denaturation	94.0	1:00
Annealing	65.0	1:00×35
Extension	72.0	1:00×35
Polishing	72.0	5:00
Hold	15.0	Hold

Table 2. Thermal Cycler Conditions

2. USER Cloning

The two PCR fragments were joined and inserted into pFC332 using the USER Cloning kit (catalog # M5505S, NEB, Ipswich, MA). To allow this, we first generated sticky ends by digesting pFC332 with *PacI* and *Nt.BbvCI* enzymes (NEB, Ipswich, MA). PacI/Nt.BbvCI digestion is an essential step in opening the pFC332 plasmid and addition of PCR products to the sticky ends. Nt.BbvCI is a nicking endonuclease that cleaves only one strand of DNA of a double-stranded DNA substrate. Digestion was achieved in 30 µl volume by combining 19 µl sterile water, 3 µl 10× CutSmart Buffer, 2 µl PacI (10 U/ul), 2 µl Nt.BbvCI (10 U/ul), and 4 µl pFC332 (313.25 ng/ul). The solution was vortexed and incubated overnight at 37℃. The digested plasmid was purified using the GeneJET PCR Purification Kit (catalog C4603, Thermo Fisher Scientific, Waltham, MA).

For USER cloning, we mixed 5 µl CutSmart buffer, 10 µl linearized pFC332 (13ng/µl), and 12.5µl of the PCR product amplified by CSN389/CSN444 PCR product, 5 µl sterile water, and 5 µl USER enzyme. Then, 15 µl of the mixture was added to three tubes containing 5 µl of the PCR product amplified by primers CSN390 and CSN445-*AUR1,* CSN445-*Tri5* or CSN445-*MGV1*. The reaction was incubated at 37℃ for two hours followed by one hour at room temperature. The reaction was stored at -20℃ until use.

3. Transformation of the USER Cloning Product into *E. coli* and Selection

The USER Cloning reaction was introduced into chemically competent *E. coli* DH5α cells. First, we added 4 µl of the corresponding USER Cloning reaction to 50 µl of competent cells. The mixture was incubated on ice for 30 minutes. Each of the tubes was heat shocked at 42℃ for 45 seconds and then kept on ice for two minutes. 250 µl of SOC was added and each solution was then left in a shaker for 1 hour at room temperature 37℃. 100 µl of each solution

containing the corresponding USER Cloning reaction was streaked and grown on ampicillin plates (*Maier, et al.*, 2005). Due to the resistance of ampicillin in the vector, the bacteria containing the targeted protospacers would only grow the bacteria with the mutated vector.

4. Plasmid Purification

For plasmid isolation, transformed *E. coli cells* were grown in 5 ml of LB/Amp over night at 37°C with shaking. 4 ml of the culture was used for plasmid isolation using the PureYield Plasmid Miniprep (Promega, Madison, WI). The overnight culture was spun at 2,500 rpm for 10 minutes and the liquid decanted off the pellet. The bacteria were resuspended in 350 µl of cell resuspension solution (CRS). After transferring to a clean 1.5 mL tube, 350 µl of cell lysis buffer was added and the solution was inverted 8 times and kept for 3 minutes. 350 µl neutralization solution was added, inverted 8 times and kept for 3 minutes. After centrifuging at maximum speed for 2 minutes, the supernatant was transferred to PureYield™ Minicolumn without disturbing the cell debris. The Minicolumn was placed in a collection tube, spun at maximum speed in a microcentrifuge for 15 seconds and the flow through discarded. 400 µl of column wash solution (CWS) was added to the Minicolumn, centrifuged at maximum speed for 30 seconds and then placed in a clean 1.5ml microcentrifuge tube. 50 µl of Elution Buffer was added, allowed to stand for 1 minute at room temperature and then centrifuged for 15 seconds to elute the plasmid DNA. The eluted plasmid was stored at DNA at –20°C. We measured the concentration of plasmids using NanoPhotometer® NP80 (Implen, Westlake Village, CA).

5. Gel Electrophoresis

We performed agarose gel electrophoresis to determine the presence of the PCR amplified products with *AUR1*, *Tri5* and *MGV1* protospacers. A 1.2% agarose gel and a 100 bp ladder (Quick-Load® Purple 100 bp DNA ladder (Fisher Scientific)) was used to compare base

pair size. Two gels were run, one after the initial protospacer synthesis PCR amplification and one after the completed USER Cloning with *E. coli* transformation.

6. Sequencing

We diluted each plasmid to 0.25 µg DNA /µl in water and sent to the Iowa State University DNA Sequencing Facility for sequencing. Sanger sequencing was performed using the BigDye chemistry. Sequencing was performed using 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). This process involved detecting fluorescently labeled DNA fragments by standard (single tube) DNA sequencing with our custom primers.

Results

1. USER Cloning

Two fragments were simplified for the USER Cloning. The first fragment contained *Aspergillus nidulans gdpA* promoter (*PgdpA*), the *tracrRNA, HDV* and the *A. nidulans trpC* terminator (*Trpc*) region from pFC334 containing the primers CSN389 and CSN390. The second fragment contained the *F. graminearum AUR1, Tri5* or *MGV1* protospacers, *tracrRNA, HDV* and *Trpc*. Figure 7 shows that the correct fragments were amplified; the first fragment was 513 base pairs and the second fragment was 412 base pairs. After verifying the fragment sizes, the PCR products were ligated to the PacI/Nt.BbvCl digested pFC332 by USER Cloning.

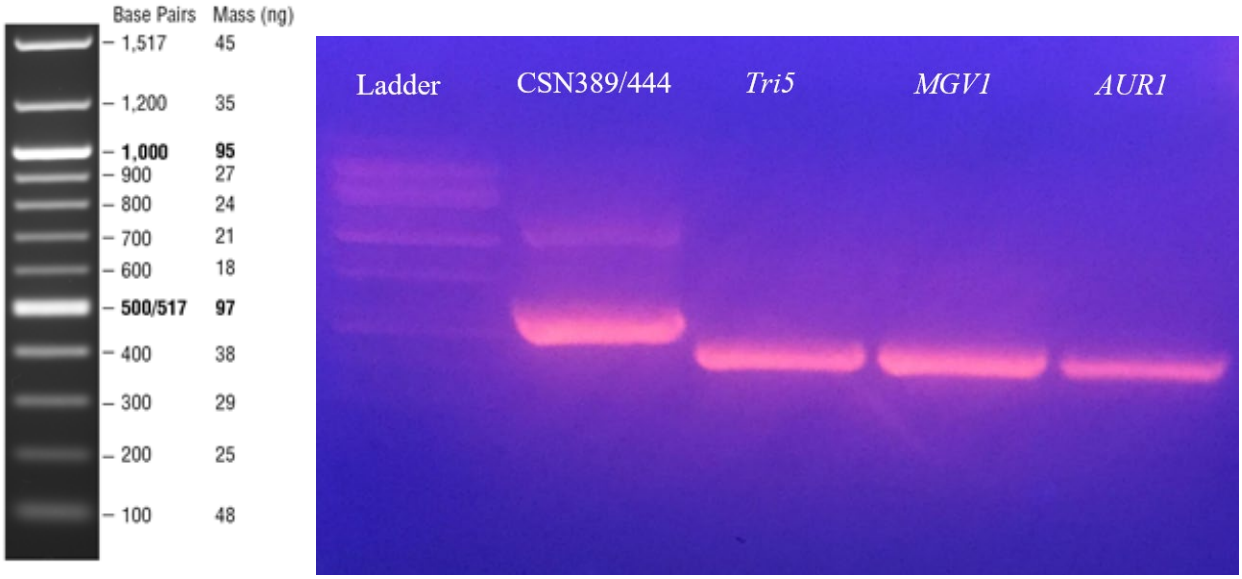

Figure 7. PCR amplification of fragments for cloning into pFC332. Ladder: Quick-Load® Purple 100 bp DNA ladder (Fisher Scientific), CSN389/CSN444 primers amplified gdpA promoter and the 5' end of hammerhead ribozyme. CSN445/CSN390 amplified *AUR1*, *Tri5*, or *AUR1* protospacer, the 3' portion of HH, tracrRNA, and trpC terminator. Fragments were separated by 1.2% agarose gel.

2. Transformation of *E. Coli*, Selection and Plasmid Purification

The USER Cloning reaction was transformed into *E. coli*. We obtained two bacterial colonies for each of *AUR1* and *Tri5* and three colonies for *MGV1*. These colonies were grown in 5 mL LB/ampicillin liquid culture. We screened the colonies for the presence of inserts using PCR. Gel electrophoresis showed that all *AUR1* and *Tri5* colonies contained the insert. For MGV1, two of the three colonies were positive (Figure 8). All bands were found to be the correct size (413 base pairs).

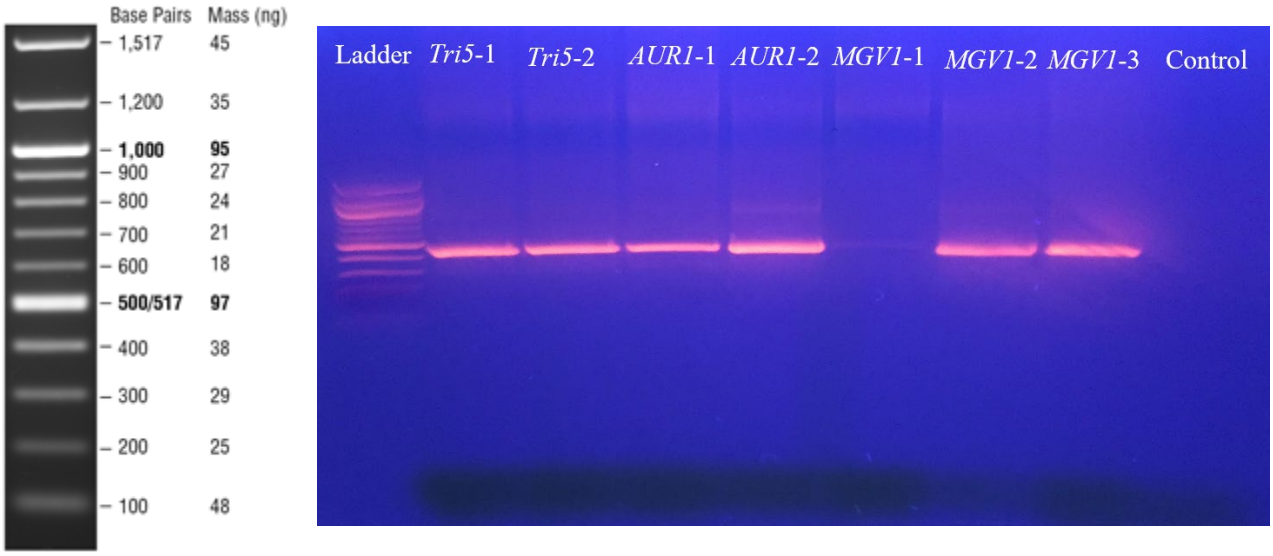

Figure 8. Detection of insert in the CRISPR/Cas9 plasmids. The region containing the protospacer was amplified by PCR using a forward primer that matches the protospacer (CSN445) and a reverse primer that matches *trpC* terminator (CSN390). PCR products were separated on 1.2% agarose. Lane 1, DNA ladder. Lane 2 and 3, *Tri-5*. Lane 4 and 5, *AUR1*. Lane 5, 6 and 7, *MGV1*. Lane 8, negative control (no DNA).

We used part of the overnight culture for plasmid purification using the PureYield Plasmid Miniprep System (Promega, Fisher Scientific). The concentration and yield of each purified plasmid are shown in Table 3. The plasmid DNA was high quality with A260/A280 of about 2.1.

Name	Concentration (ng/µl)	Yield/50 µl (µg)
Tri5-1	245.10	12,255
Tri5-2	239.75	11,987.5
AUR1-1	481.40	24,070
AUR1-2	380.60	19,030
MGV1-1	427.25	21,362.5
MGV1-2	470.50	23,525
MGV1-3	595.55	29,777.5

Table 3. NanoPhotometer® results for *Tri5*, *AUR1*, and *MGV1* plasmid concentrations

4. Sequencing

The CRISPR/Cas9 plasmids we constructed were sequenced to verify the identity of the protospacer sequences (*AUR1, Tri5,* or *MGV1*) within the sgRNA (containing *HH* and *HDV*), *gdpA* promoter and *trpC* terminator. All the plasmids contained the correct sequence (Appendix) so that plasmids can be used for mutating *F. graminearum* in future experiments.

Discussion

The gel electrophoresis for synthesis of the protospacer sequences, showed a positive primer result from the original PCR screening. The bands also showed a range of 413 base pairs which is indicative of the length of the expected sequencing of the sgRNA based on the full genome (appendix). This provided successful fragment development for USER Cloning.

The USER Cloning combining the pFC332 plasmid with the primer fragments is an essential step for transformation. Combining the Cas9 enzyme with the gene fragments is the main step for creating a vector that allows for CRISPR/Cas9 modification once inserted into *F. graminearum* to mutate gene function.

The USER product was able to be successfully transformed into *E. coli* bacteria which was able to be observed from the purified plasmids from the colonies. The first observation determined for successful transformation was by growing the bacteria on ampicillin plates which killed all bacteria except those resistant to ampicillin, in this case our modified CRISPR/Cas9 pFC332 plasmid which now had the targeted genes, *AUR1, Tri5* and *MGV1*. We also found this to be true in our liquid cultures that also contained ampicillin.

Finally, after plasmid purification, by running a gel we were able to show our transformed and mutated vectors in *E. coli* colonies next to a negative control with no DNA.

There was no band on the negative control which confirmed that the amplified products were not due to contamination due to the PCR reagents. Our positive bands also showed a base pair range at 413 base pairs, which is again the length of the expected sequencing for the sgRNA from the full genome (appendix). Based on previous studies, formation of vectors with these protocols was expected.

In conclusion, our results confirmed the presence of the protospacers for *AUR1*, *Tri5*, and *MGV1* genes by PCR and gel electrophoresis and USER Cloning was successful and confirmed by sequencing. Going forward, The CRISPR/Cas9 plasmids will be introduced into *F. graminearum* to knock out *AUR1*, *Tri5*, and *MGV1* genes. Barley heads (inflorescence) will be infected using mutagenized and wild type *F. graminearum* and severity of infection will be compared. We anticipate that the mutation would compromise the ability of *F. graminearum* to infect its host and ultimately promote healthy crop growth based on the CRISPR/Cas9 system. Scab is a devastating disease of cereals. Its economic impact in the U.S. is significant. Since 1990, it is estimated wheat and barley farmers in the United States have lost over $2.6 billion dollars due to FHB epidemics (Windels CE, 2000). *F. graminearum* mutagenesis not only provides obvious economic benefits, but also healthy and safe agriculture development for the future.

Conclusion

F. graminearum is a devastating fungal disease on many cereal crops. By genetically modifying this fungus, the ability of the plant to infect its host can be compromised. The goal of this study was to construct CRISPR/Cas9 vectors containing guide RNA sequences to mutate three important genes in *F. graminearum* genome that are essential for infection: *Tri5*, *MGV1* and *AUR1*. Our results showed confirmed sequences for the mutated plasmid vectors. Although these data do not show direct impact on how these mutated vectors affect *F. graminearum*, this is an essential step to know the complete process for developing these vectors to eventually transform vectors into the fungus in future studies and observe results on barley plants for agricultural development.

This study involved some development of the best way to produce positive PCR amplification and gel electrophoresis, USER Cloning and *E. coli* transformation. Because there was little known resistance against *F. graminearum* and few studies on how these specific genes affect infection, this is an important study to develop these modified vectors and ultimately observe how they affect infection or disturb infection in barley compared to the wild type. These results show promise for not only the accurate and effective development of modified vectors, but also in genetically protecting cereal crops in an agriculture setting.

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Appendix

PCR Primer sequences (protospacer sequence is shaded)

CSN445-*AUR1*: 5'-
AGTAAGC/ideoxyU/CGTCTTTCCAGACGCAGCTGACTTGTTTTAGAGCTAGAAATAG
CAAGTTAAA-3′

CSN445-*Tri5*: 5'-
AGTAAGC/ideoxyU/CGTCGAACCTTATCCGTAGCACTAGTTTTAGAGCTAGAAATAG
CAAGTTAAA-3′

CSN445-*MGV1*: 5'-
AGTAAGC/ideoxyU/CGTCCGTACCGTGCCCGGCCAAAGGTTTTAGAGCTAGAAATAG
CAAGTTAAA-3′

CSN444: 5'-
AGCTTAC/ideoxyU/TCGTTTCGTCCTCACGGACTCATCAGCGAAGGCGGTGATGTCT
GCTCAAGCG-3′

CSN390: 5′-GGTCTTAA/ideoxyU/GAGCCAAGAGCGGATTCCTC-3′

CSN389: 5′-GGGTTTAA/ideoxyU/GCGTTAAGCTCCCTAATTGGC-3′

Sequencing Primer

gpdAin11F 5′-GCTACATCCATACTCCATCCC-3′

Sequence of Region Containing Protospacer

AUR (protospacer sequence is shaded):

NNNNNNNNNNNNNACTTTANTTCGAGCTTTCCCACTTCATCGCAGCTTGACTAACAGCT
ACCCCGCTTGAGCAGACATCACCGCCTTCGCTGATGAGTCCGTGAGGACGAAACGAAGT
AAGCTCGTCTTTCCAGACGCAGCTGACTTGTTTTAGAGCTAGAAATAGCAAGTTAAAAT
AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCAT
GGTCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGAC
TGATTTAATAGCTCCATGTCAACAAGAATAAAACGCGTTTCGGGTTTACCTCTTCCAGA
TACAGCTCATCTGCAATGCATTAATGCATTGGACCTCGCAACCCTAGTACGCCCTTCAG
GCTCCGGCGAAGCAGAAGAATAGCTTAGCAGAGTCTATTTTCATTTTCGGGAGACGAGA
TCAAGCAGATCAACGGTCGTCAAGAGACCTACGAGACTGAGGAATCCGCTCTTGGCTCA
TTAAGACCTCAGCCGAGACAGCAGAATCACCGCCCAAGTTAAGCCTTTGTGCTGATCAT
GCTCTCGAACGGGCCAAGTTCGGGAAAAGCAAAGGAGCGTTTAGTGAGGGGCAATTTGA
CTCACCTCCCAGGCAACAGATGAGGGGGGCAAAAAGAAAGAAATTTTCGTGAGTCAATA

TGGATTCCGAGCATCATTTTCTTGCGGTCTATCTTGCTACGTATGTTGATCTTGACGCT
GTGGATCAAGCAACGCCACTCGCTCGCTCCATCGCAGGCTGGTCGCAGACAAATTAAAA
GGCGGCAAACTCGTACAGCCGCGGGGTTGTCCGCTGCAAAGTACAGAGTGATAAAAGCC
GCCATGCGACCATCAACGCGTTGATGCCCAGCTTTTTCGATCCGAGAATCCACCGTAGA
GGCGATAGCAAGTAAAGAAAAGCTAAACAAAAAAAAATTTCTGCCCCTAAGCCATGAAA
ACGAGATGGGGTGGAGCAGAACCAAGGAAAGAGTCGCGCTGGGCTGCCGTTCCGGAAGG
TGTTGTAAAGGCTCGACGCCCAAGGTGGGAGTCTAGGAGAAGAATTTGCATCGGGAGTG
GGGCGGGTTACCCCTCCATATCCAATGACAGATATCTACCAGCCAAGGGTTTGAACCCG
CCCGCTTAATCGTCGTCCNCGCTTGCCCCTCCAAAAAAGGATTTCCCCTCCCCCTCCAC
AAAATTTTCTTTCCCTTCCTCTCCTTGGCCGNTTCAGAACGAAAATTTTCCCTTCCCCC
GCC

Tri5 (protospacer sequence is shaded):

NNNNNTNNNNTTTTNACTTTNNNTTCGAGCTTTCCCACTTCATCGCAGCTTGACTAACA
GCTACCCCGCTTGAGCAGACATCACCGCCTTCGCTGATGAGTCCGTGAGGACGAAACGA
AGTAAGCTCGTCGAACCTTATCCGTAGCACTAGTTTTAGAGCTAGAAATAGCAAGTTAA
AATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGG
CATGGTCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGG
GACTGATTTAATAGCTCCATGTCAACAAGAATAAAACGCGTTTCGGGTTTACCTCTTCC
AGATACAGCTCATCTGCAATGCATTAATGCATTGGACCTCGCAACCCTAGTACGCCCTT
CAGGCTCCGGCGAAGCAGAAGAATAGCTTAGCAGAGTCTATTTTCATTTTCGGGAGACG
AGATCAAGCAGATCAACGGTCGTCAAGAGACCTACGAGACTGAGGAATCCGCTCTTGGC
TCATTAAGACCTCAGCCGAGACAGCAGAATCACCGCCCAAGTTAAGCCTTTGTGCTGAT
CATGCTCTCGAACGGGCCAAGTTCGGGAAAAGCAAAGGAGCGTTTAGTGAGGGGCAATT
TGACTCACCTCCCAGGCAACAGATGAGGGGGGCAAAAAGAAAGAAATTTTCGTGAGTCA
ATATGGATTCCGAGCATCATTTTCTTGCGGTCTATCTTGCTACGTATGTTGATCTTGAC
GCTGTGGATCAAGCAACGCCACTCGCTCGCTCCATCGCAGGCTGGTCGCAGACAAATTA
AAAGGCGGCAAACTCGTACAGCCGCGGGGTTGTCCGCTGCAAAGTACAGAGTGATAAAA
GCCGCCATGCGACCATCAACGCGTTGATGCCCAGCTTTTTCGATCCGAGAATCCACCGT
AGAGGCGATAGCAAGTAAAGAAAAGCTAAACAAAAAAAAATTTCTGCCCCTAAGCCATG
AAAACGAGATGGGGTGGAGCAGAACCAAGGAAAGAGTCGCGCTGGGCTGCCGTTCCGGA
AGGTGTTGTAAAGGCTCGACGCCCAAGGTGGGAGTCTAGGAGAAGAATTTGCATCGGGA
GTGGGGCGGGTTACCCCTCCATATCCAATGACAGATATCTACCAGCCAAGGGTTTGAAC
CCGCCCCGCTTAGTCGTCGTCCCTCGCTTGCCCCTTCCTAAAAAGGATTTCCCCTCCCC
CCCCCCCAAAAATTTTCTTTCCCTTCCNTCCCTTGGCCCGCTTCNGAAAGNNNAATTTC
CNNNCCCCCNNCTTCTCCCCCCCCACCCTTTTTTTTTACCCATTCCNNGGAAAATTTTT
TGGGCCCACCCCCCCTCCNNCTTTTNNNNCCCCGAAATTTTTGAAAACTTTTCTCCCTT
TTTTTTTTCCA

MGV1 (protospacer sequence is shaded):

NNNNNNNNNTNNAACTTTCAGTTCGAGCTTTCCCACTTCATCGCAGCTTGACTAACAGC
TACCCCGCTTGAGCAGACATCACCGCCTTCGCTGATGAGTCCGTGAGGACGAAACGAAG
TAAGCTCGTCCGTACCGTGCCCGGCCAAAGGTTTTAGAGCTAGAAATAGCAAGTTAAAA
TAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTGGCCGGCA

TGGTCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCGAATGGGA
CTGATTTAATAGCTCCATGTCAACAAGAATAAAACGCGTTTCGGGTTTACCTCTTCCAG
ATACAGCTCATCTGCAATGCATTAATGCATTGGACCTCGCAACCCTAGTACGCCCTTCA
GGCTCCGGCGAAGCAGAAGAATAGCTTAGCAGAGTCTATTTTCATTTTCGGGAGACGAG
ATCAAGCAGATCAACGGTCGTCAAGAGACCTACGAGACTGAGGAATCCGCTCTTGGCTC
ATTAAGACCTCAGCCGAGACAGCAGAATCACCGCCCAAGTTAAGCCTTTGTGCTGATCA
TGCTCTCGAACGGGCCAAGTTCGGGAAAAGCAAAGGAGCGTTTAGTGAGGGGCAATTTG
ACTCACCTCCCAGGCAACAGATGAGGGGGGCAAAAAGAAAGAAATTTTCGTGAGTCAAT
ATGGATTCCGAGCATCATTTTCTTGCGGTCTATCTTGCTACGTATGTTGATCTTGACGC
TGTGGATCAAGCAACGCCACTCGCTCGCTCCATCGCAGGCTGGTCGCAGACAAATTAAA
AGGCGGCAAACTCGTACAGCCGCGGGGTTGTCCGCTGCAAAGTACAGAGTGATAAAAGC
CGCCATGCGACCATCAACGCGTTGATGCCCAGCTTTTTCGATCCGAGAATCCACCGTAG
AGGCGATAGCAAGTAAAGAAAAGCTAAACAAAAAAAAATTTCTGCCCCTAAGCCATGAA
AACGAGATGGGGTGGAGCAGAACCAAGGAAAGAGTCGCGCTGGGCTGCCGTTCCGGAAG
GTGTTGTAAAGGCTCGACGCCCAAGGTGGGAGTCTAGGAGAAGAATTTGCATCGGGAGT
GGGGCGGGTTACCCCTCCATATCCAATGACAGAAATCTACCAGCCAAGGGTTTGAGCCC
GCCCGCTTAGTCGTCGTCCTCGCTTGCCCCTCCATAAAAGGATTTCCCCTCCCCTCCCC
AAAATTTTCTTTCCCTTCCCTCCCTGGCCCGCTTCAGAACGAAAATCTTCCCTTCCCCC