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# Construction of a target-specific CRISPR-Cas9 vector for the purpose of introducing mutations into the FUM1 gene of *Fusarium verticillioides*

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# CONSTRUCTION OF A TARGET-SPECIFIC CRISPR-CAS9 VECTOR FOR THE PURPOSE OF INTRODUCING MUTATIONS INTO THE FUM1 GENE OF *FUSARIUM VERTICILLIOIDES*

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**Abstract**

In molecular biology, CRISPR-Cas9 is a useful gene editing tool that takes advantage of a powerful cleaving enzyme and DNA sequences found in prokaryotes. In this study, a target-specific CRISPR-Cas9 vector containing an RNA-guided Cas9 nuclease specific for the FUM1 gene was constructed for the purpose of introducing mutations into the FUM1 gene of *Fusarium verticillioides*. Plasmids pFC332 and pFC334 were isolated. A guide RNA sequence was designed and amplified using Polymerase Chain Reaction (PCR) with pFC334 as the template. Sticky ends were created through restriction enzyme digestion of pFC332. These pieces were combined using Uracil-Specific Excision Reagent (USER) fusion, and the resulting vector was transformed into competent *E. coli* cells. Colonies containing the constructed vector were selected, and PCR was performed for amplification. The results were purified and stored at -40°C. This constructed vector can be used for the specific mutation of the FUM1 gene of *Fusarium verticillioides.*

**Introduction**

CRISPR-Cas9 is a gene editing tool that is becoming an important player in genetic research. CRISPRs, or clustered regularly interspaced palindromic repeats, are short segments of DNA that serve as an immune mechanism in bacteria and archaea. Cas9, or CRISPR associated protein 9, is a large protein that was found to be imperative in the defense against viral invasion in prokaryotes. It is an RNA-guided DNA endonuclease that scientists have recently taken advantage of for genome engineering. Use of this system requires a single guide RNA that includes a sequence of 20 nucleotides that align with the target DNA site and a double-stranded sequence that binds to Cas9. A PAM (protospacer adjacent motif) site is a 3-nucleotide sequence

of DNA that is essential for the proper binding of the Cas9 protein. As long as the segment of target DNA is adjacent to a PAM site, the system can be manipulated to work with any target. (Doudna and Charpentier, 2014).

*Fusarium verticillioides* is one of the most commonly seen species of *Fusarium* and is a known producer of fumonisin. *Fusarium* is a plant pathogenic species of fungi that commonly infects maize, among other crops (Facchini et al., 2017), and fumonisins are known carcinogenic mycotoxins that were first isolated from a species of *F. verticillioides* in 1988. *F. verticillioides* is one of the most important producers of fumonisins, since it is geographically widespread and produces high levels of the toxin (Rheeder et al., 2002). Understanding the cause of pathogenicity brings economic and human health benefits. It gives scientists guidance for ways to target this species in order to make it less virulent. In addition, it allows insight for the best ways to protect crops from this pathogen, in turn protecting humans from a known carcinogen.

A system has been developed for the use of CRISPR-Cas9 in filamentous fungi. Four vectors were prepped with commonly used selection markers, in addition to scaffolding for guide RNA synthesis, so that it can be adapted to be used in different species and for different target genes. A gene host can be transformed with a single plasmid that contains the components of the CRISPR-Cas9 system, the Cas9 nuclease, a gene-specific guide RNA, and the desired selection marker (Nodvig et al., 2015). In the spring of 2019, this system was used for the silencing of the yA gene in *Aspergillus nidulans*. This initial study served as an introduction into the use of CRISPR-Cas9 systems in filamentous fungi and provided a basis for the mutation of the FUM1 gene in *Fusarium verticillioides.* In addition, the protocols used for the collection of spores, production of protoplasts, and PEG-mediated transformation used for *A. nidulans* can be followed closely in the use of *F. verticillioides.*

## Hypothesis

In this study, a target-specific CRISPR-Cas9 vector has been constructed for the mutation of the FUM1 gene of *Fusarium verticillioides.* The successful construction of this vector was detected through the use of a Polymerase Chain Reaction (PCR) including the original guide RNA primers and the completed vector isolated from transformed competent cells as the template. It is hypothesized that if this CRISPR-Cas9 vector containing the FUM1 gene specific guide RNA and a hygromycin resistance selection marker is transformed into protoplasts of *Fusarium verticillioides* using a PEG-mediated method, then the FUM1 gene will be silenced in the colonies that successfully grow in a hygromycin environment. This silencing can be determined by using PCR analysis specific to the FUM 1 gene.

## Methodology

### *Introduction of mutations into the yA gene of Aspergillus nidulans*

Plasmid pFC334 was isolated from a culture that already contained it using the Thermo Scientific GeneJET Plasmid Miniprep Kit. The protocol provided with the kit was followed without any changes. The resulting plasmid DNA was run on a 1% agarose gel and used later for the transformation of protoplasts. When studying an agarose gel, it is important to note that the size of the DNA fragment determines how far it will run. Larger fragments will travel further on the gel than smaller fragments. Standard ladders are run with samples and used to estimate the size of the unknown fragments. Ethidium bromide is used to enable visibility of the DNA bands.

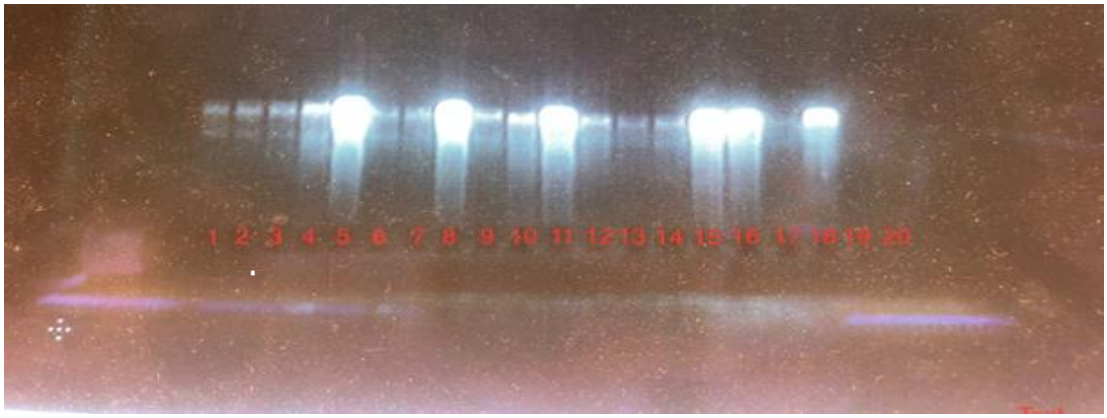

**Figure 1: The gel for the isolation of pFC334 plasmid DNA, lanes are numbered from left to right.**

| **Well #** | |
|---|---|
| 1-9 | pFC334 plasmid DNA |
| 10-18 | pFC332 plasmid DNA |
| 19 | Lambda-HindIII Standard |
| 20 | Blank |

**Table 1: Table for the gel run for isolation of pFC334 plasmid DNA.**

In order to produce protoplasts of *Aspergillus nidulans*, spores were first collected. Spores were collected from colonies of *A. nidulans* by adding and spreading Tween in a sterile environment using hockey sticks and ethanol. The resulting disrupted spores were collected and filtered through miracloth and rinsed with the addition of more Tween. This resulting solution was vortexed and used for the production of protoplasts. The images taken of the microscope

samples show that there were indeed protoplasts during this step of the experiment. Protoplasts were essential for the transformation, because without them the presence of a cell wall inhibits the ability of a plasmid DNA to be inserted.

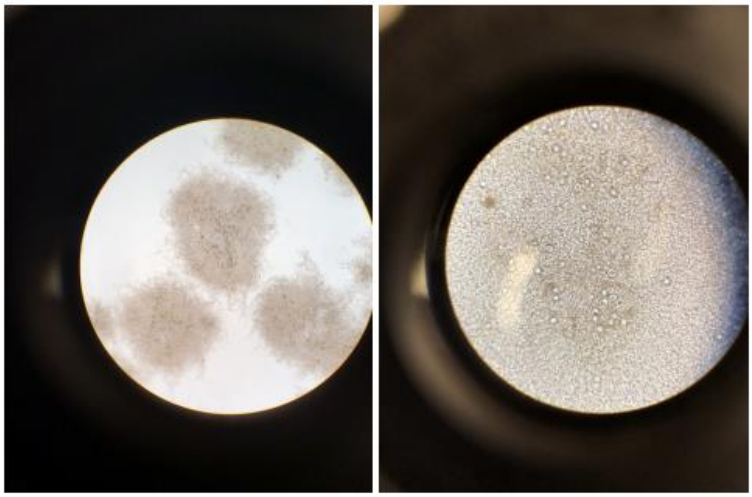

**Figure 2: Samples under microscope during protoplast production. Left image is viewed at 100x magnification, right is viewed at 400x magnification.**

Spores were inoculated in 20 mLs of YT medium containing arginine and biotin as growth supplements while incubating and shaking overnight. Hyphae was harvested by filtration through miracloth and washed with growth medium. Hyphae was scraped with a spatula and resuspended in 8 mLs of YT solution. 8 mLs of the protoplasting solution were added after filter sterilization. This solution was incubated with gentle shaking for two hours. The undigested hyphal material was removed by layering the protoplasting mixture onto a 1.2 M sucrose cushion and centrifuging in a clinical centrifuge. Protoplasts were collected from the top of the sucrose cushion and placed in a centrifuge tube with equal volume 0.6 M KCl for centrifugation. The protoplasts formed a loose pellet, which was resuspended in 2 mL of 0.6 M KCl and transferred to two new microcentrifuge tubes. Protoplasts were again pelletized and resuspended in 0.6 M

KCl 3 additional times, then the pellets were finally resuspended in 0.5 mL of 0.6 M KCl and 50 mM CaCl2. Throughout the steps of this procedure, samples were viewed under a microscope to monitor progress of protoplast production.

Ten µL of isolated pFC334 DNA were added to 100 µL protoplast suspension and mixed thoroughly. 50 µL of PEG solution were added and the solution was vortexed and placed in an ice bath for 25 minutes. 1 mL of PEG solution was added, mixed, and put in an ice bath for 25 minutes. 100 µL were added to minimal media plates containing biotin and arginine and left to grow. Resulting yellow colonies were isolated and streaked on nonselective plates and left to grow as well. Since this plasmid is selective for arginine, the ability to grow colonies on the plates containing arginine confirmed that the plasmid had indeed transformed the protoplasts. The yellow colonies that grew from the transformation represented a successful silencing of the yA gene in *A. nidulans*, since they were lacking the green pigment. Yellow colonies were then chosen and grown on non-selective plates, and this resulted in rapid yellow colony growth. This shows that the phenotype is stable without the presence of the plasmid.

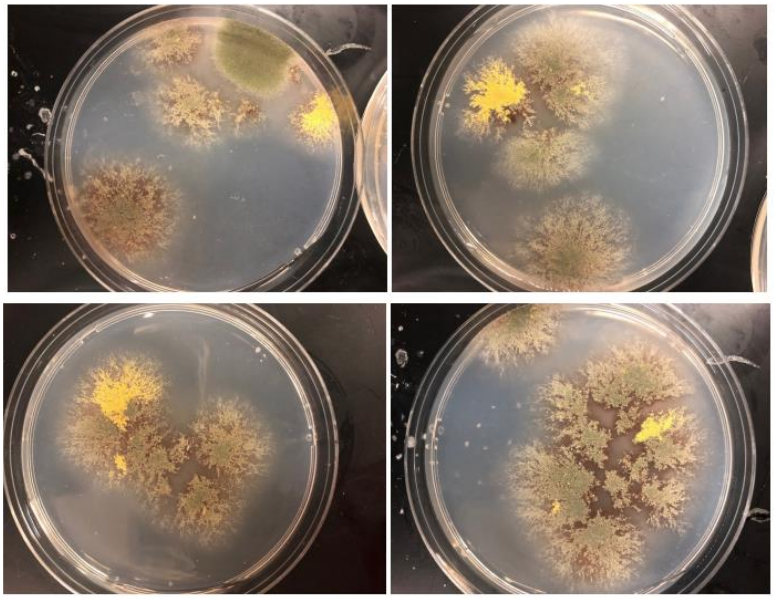

**Figure 3: Resulting colonies from transformation of *A. nidulans* protoplasts with pFC334 plasmid DNA grown on arginine selective plates.**

**Figure 4: Isolated yellow colonies from transformation streaked on a non-selective plate.**

***Construction of the FUM1 Specific Vector***

The biggest difference between the transformation performed in *Aspergillus nidulans* and that in *Fusarium verticillioides* is the construction of a CRISPR-Cas9 vector specific for the FUM1 gene of *F. verticillioides.* The first step in construction was isolating plasmids pFC332 and pFC334 (Nødvig et al, 2015). These isolations were performed using the Thermo Scientific GeneJET Plasmid Miniprep Kit, and the protocol provided with the kit was followed without any changes. Plasmid pFC332 contains the gene for hygromycin resistance, which was the chosen selection marker for this study. Plasmid pFC334 contains the gRNA scaffold, allowing for the introduction of a guide RNA specific for FUM1. The results of these isolations were analyzed by gel electrophoresis on a 1% agarose gel. This isolation was successful, since the bands represented the expected sizes for the plasmids. Plasmid pFC332 was around 15,000 base pairs and pFC334 was around 17,000 base pairs. Both plasmids were characterized with a restriction enzyme digest using EagI. This restriction enzyme was chosen because it has 5 cut sites on

pFC332 and only 2 sites of pFC334. This would allow for the characterization and distinction of the two plasmids, but a successful image was not obtained.

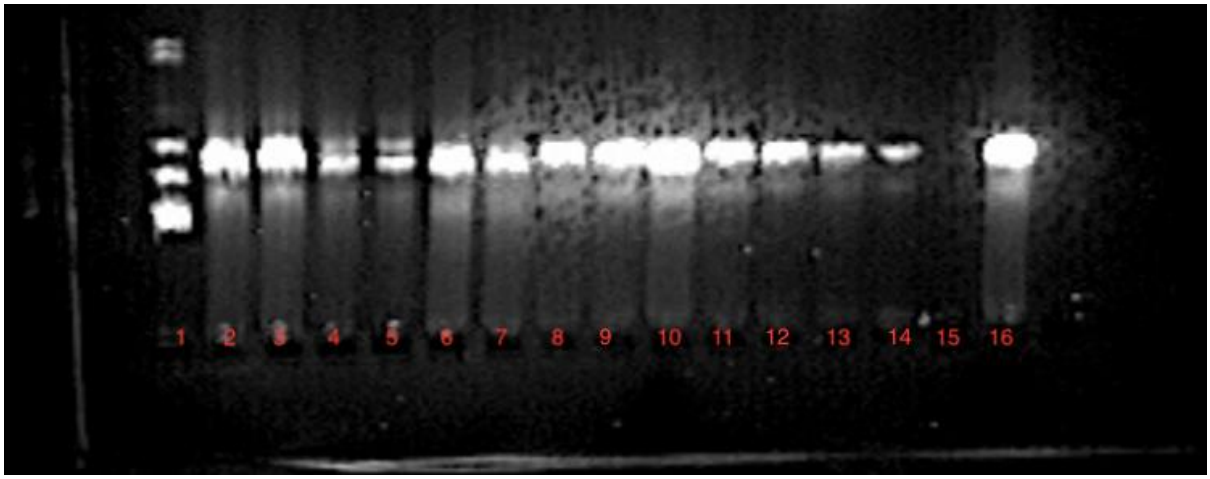


**Figure 5: The gel for the isolation of pFC332 and pFC334 plasmid DNA, lanes are numbered from left to right.**

| **Well #** | |
|---|---|
| 1 | Lambda-HindIII Standard |
| 2-3, 8-10 | pFC332 plasmid DNA |
| 6-7, 14-16 | pFC334 plasmid DNA |

**Table 2: Table for the gel run for isolation of pFC332 and pFC334 plasmid DNA.**

Once the plasmids were isolated, a guide RNA specific to the FUM1 gene of *Fusarium verticillioides* was designed. The Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool was utilized in the design of the guide RNA sequence. This tool was chosen because it could compare the sequence of a target gene to the entire genome, and it had the genome of *Fusarium*

*verticillioides* available in its database. The Broad Institute has previously sequenced the complete genome of *F. verticillioides* and is where the sequence of the FUM1 gene was gathered. The FUM1 gene sequence was compared to the entire genome of *F. verticillioides* using the guide RNA design tool, and a resulting guide RNA sequence with the fewest number of offsite targets and a location towards the middle of the gene was chosen. Offsite targets could cause the CRISPR-Cas9 system to bind and cleave DNA in different areas of the genome, and a target sequence too early or late within the FUM1 gene could result in no impact on the gene expression. This is why these parameters were taken into consideration. Once a sequence was chosen, it was used in the building of PCR primers based on the example of primers CSN414 and CSN416 (Nødvig et al, 2015). The resulting primers, FUM1 Right Forward Primer 1 (5'-AGTAAGCUCGTCATTCTCACCGACCATGTCTGTGGGTTTTAGAGCTAGAAATAGCAAGTT-3'), and FUM1 Left Reverse Primer 1 (5'-AGCTTACUCGTTTCGTCCTCACGGACTCATACCTTCTCCGGTGATGTCTGCTCAAGCG-3'), were ordered from Integrated DNA Technologies along with primers CSN389 (5'-GGGTTTAAUGCGTAAGCTCCCTAATTGGC-3') and CSN390 (5'-GGTCTTAAU GAGCCAAGAGCGGATTCCTC-3') (Nødvig et al, 2015).

| Primer | Sequence |
|---|---|
| CSN414 | 5'-AGCTTACUCGTTTCGTCCTCACGGACTCATCAGGGCGACCGGTGATGTCTGCTCAAGCG-3' |
| CSN416 | 5'-AGTAAGCUCGTCGGCGACATGTTGAAGAACCGGTTTTAGAGCTAGAAATAGCAAGTTAAA-3' |
| FUM1 Left Reverse Primer 1 | 5'-AGCTTACUCGTTTCGTCCTCACGGACTCATACCTTCTCCGGTGATGTCTGCTCAAGCG-3' |
| FUM1 Right Forward Primer 1 | 5'-AGTAAGCUCGTCATTCTCACCGACCATGTCTGTGGGTTTTAGAGCTAGAAATAGCAAGTT-3' |
| CSN389 | 5'-GGGTTTAAUGCGTAAGCTCCCTAATTGGC-3' |
| CSN390 | 5'-GGTCTTAAU GAGCCAAGAGCGGATTCCTC-3' |

**Table 3: Sequences for PCR primers used**

Once the constructed primers arrived from IDT, PCR reactions were performed. Two separate reactions were performed: one using plasmid pFC334 as a template with FUM1 Right Forward Primer 1 and CSN390 and the other using pFC334 as a template with FUM1 Left Reverse Primer and CSN389. Sequences for primers CSN389 and CSN390 were provided by Nødvig et al (2015). The results of these PCR reactions were analyzed through gel electrophoresis on a 1% agarose gel. The resulting fragments supported the successful amplification using these specific primers and the introduction of the specific guide RNA for the FUM1 gene.

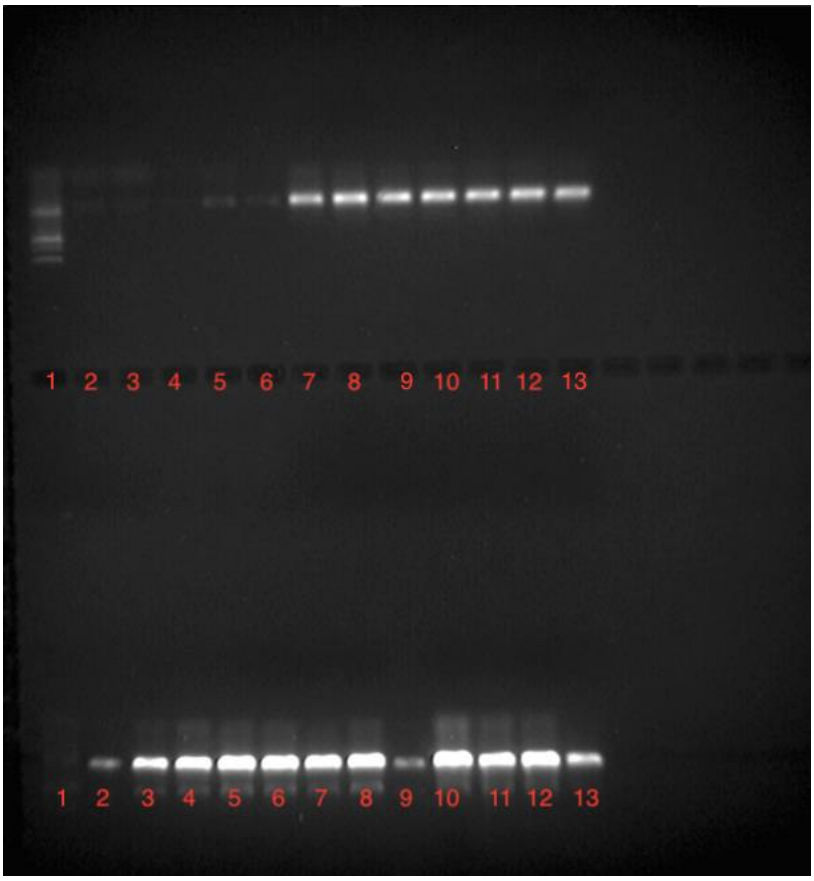


**Figure 6: The gel for the PCR reactions of plasmid pFC334, lanes are numbered from left to right, bottom to top.**

| **Well #** | |
|---|---|
| Bottom 1 | 100 Base Pair Ladder Standard |
| Bottom 2-13 | Primers FUM1 Right Forward Primer 1 and CSN390 |
| Top 1 | pFC334 plasmid DNA |
| Top 2-13 | Primers FUM1 Left Reverse Primer 1 and CSN389 |

**Table 4: Table for the gel run for the PCR reactions of plasmid pFC334.**

In order to construct the vector, plasmid pFC332 was digested with the restriction enzymes PACI and Nt.BbvCI. For the restriction enzyme digest, 15 µL of plasmid pFC332 were combined with 1 µL PACI, 1 µL Nt.BbvCI, 2 µL cutsmart buffer, and 1 µL of distilled water. This reaction was combined in a microcentrifuge tube and left to incubate overnight at 37°C. The next day, an additional microliter of Nt.BbvCI was added to the mixture and left to incubate for one hour. This step was included to increase the efficiency of the enzyme. This reaction was purified using the Thermo Scientific GeneJET Gel Extraction and DNA Cleanup Micro Kit. The protocol provided with the kit was followed without any modifications. This restriction enzyme digest provided sticky ends, which are important for the introduction of the guide RNA using Uracil-Specific Excision Reagent (USER) fusion.

USER fusion is a powerful technique that allows the cloning and ligation of several PCR fragments into a vector in one relatively quick and simple step. In this study, USER fusion allowed for the construction of the vector in one step, ligating the two plasmid pFC334 PCR products with the purified restriction enzyme digest of plasmid pFC332 (New England Biolabs). The PCR products were diluted 1:5 in distilled water. 1 µL of Thermolabile USER II Enzyme was combined with 1 µL of each dilute PCR product, 1 µL of the purified restriction enzyme digest plasmid, and 6 µL of cutsmart buffer. This mixture was incubated for 20 minutes at 37℃, followed by 25 minutes at room temperature. It was then directly transformed into competent *E. coli* cells.

For the transformation of *E. coli*, all 10 µL of the USER fusion reaction were added to the tube of competent cells and left to chill on ice for 30 minutes. The tube was heat shocked at 42℃ for 90 sec before being chilled for another 90 sec. 800 µL SOC were added and the tube was incubated in a 37℃ heat bath at 225 RPM for one hour. The results were spread in a sterile environment with a hockey stick and flaming alcohol on agar plates containing ampicillin. In this step of the experiment, ampicillin resistance was used as the selection marker. Only cells that had been transformed with the constructed vector would survive in the presence of ampicillin, and these colonies were isolated and amplified using colony PCR. Primers CSN389 and CSN390 were used as primers in this PCR reaction in order to amplify the constructed vector.

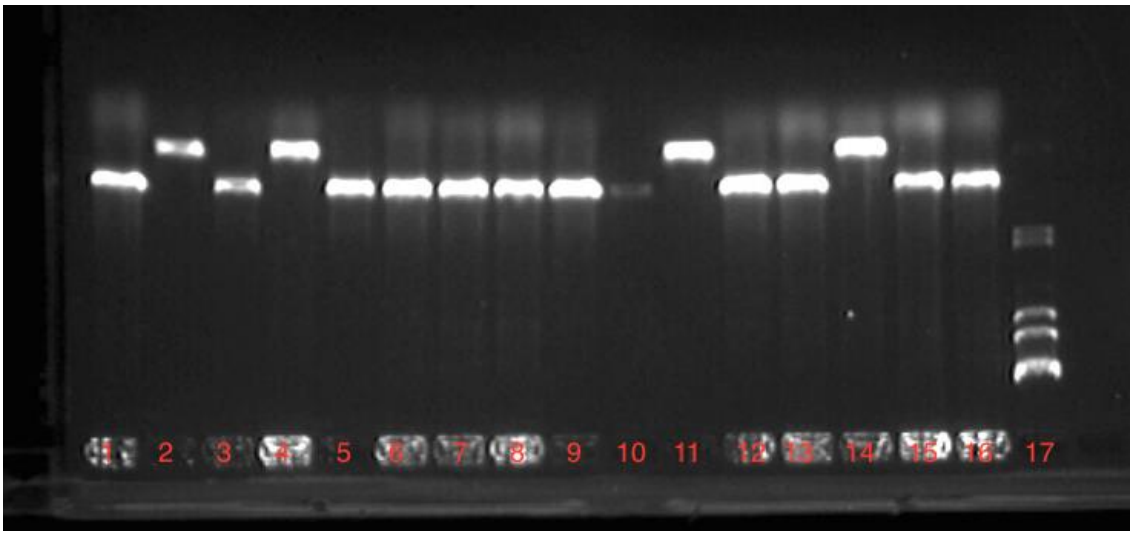


**Figure 7: The gel for colony PCR using primers CSN389 and CSN390, lanes are numbered from left to right.**

| **Well #** | |
|---|---|
| 1, 3, 5-10, 12-13, 15-16 | Successful USER fusion and transformation |
| 17 | Lambda-HindIII Standard |

**Table 5: Table for the gel run for colony PCR using primers CSN389 and CSN390.**

The Thermo Scientific GeneJET Plasmid Miniprep Kit was used for purification of the amplified plasmids that represented successful USER fusion, and the protocol was followed without any changes. The results of the isolation were analyzed by gel electrophoresis on a 1% agarose gel. The corresponding fragments suggest that the isolation of this vector was successful. This purified vector will be used directly in the transformation of *Fusarium verticillioides.*


**Figure 8: The gel for the isolation of the constructed FUM1-specific CRISPR-Cas9 vector, lanes are numbered from left to right.**

| **Well #** | |
|---|---|
| 1 | Lambda-HindIII Standard |
| 10-17 | Isolated FUM1-Specific CRISPR-Cas9 Vector |

**Table 6: Table for the gel run for the isolation of the constructed FUM1-specific CRISPR-Cas9 vector.**

Another PCR reaction was performed to check that both PCR fragments had been incorporated into the vector. The primers used in the initial PCR of plasmid pFC334 were used in two separate reactions, and the results were analyzed using gel electrophoresis on a 1% agarose gel. The expected fragments were visible, supporting the successful construction of the FUM1 specific vector.

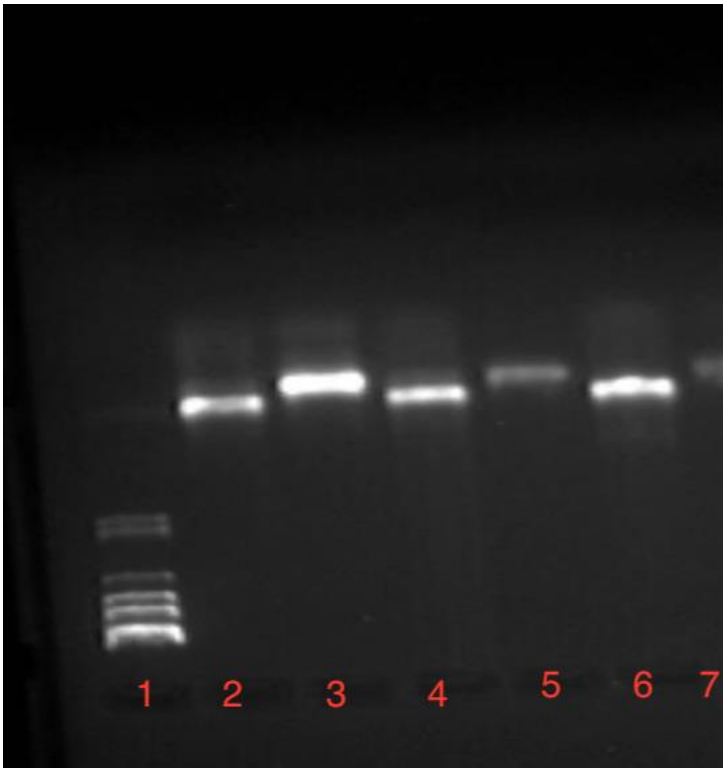


**Figure 9: The gel for the PCR reactions of the constructed FUM1-specific CRISPR-Cas9 vector, lanes are numbered from left to right.**

| **Well #** | |
|---|---|
| 1 | Lambda-HindIII Standard |
| 2, 4, 6 | Primers FUM1 Left Reverse Primer 1 and CSN389 |
| 3, 5, 7 | Primers FUM1 Right Forward Primer 1 and CSN390 |

**Table 6: Table for the gel run for the PCR reaction of the constructed FUM1-specific CRISPR-Cas9 vector.**

Due to time constraints in response to the COVID-19 outbreak, this is as far as the research was able to be completed. The subsequent sections reflect the protocol that was intended for the final steps of the procedure, and the anticipated results for if the project is ever able to be completed.

### *Collection of Spores*

In order to produce protoplasts of *Fusarium verticillioides*, spores will first be collected. Spores will be collected from colonies of *F. verticillioides* by adding and spreading 0.01% Tween 80 in a sterile environment using hockey sticks and ethanol. The resulting disrupted spores will be collected and filtered through miracloth and rinsed with the addition of more 0.01% Tween 80. The resulting solution will be vortexed and used for the production of protoplasts (Szewczyk, 2006).

### *Production of Protoplasts*

Spores will be inoculated in 20 mLs of fusarium medium and incubated and shaken overnight at 30℃. Hyphae will be harvested by filtration through miracloth and washed with growth medium. Hyphae will be scraped with a spatula and resuspended in 8 mLs of CM solution. 8 mLs of the protoplasting solution will be added after filter sterilization. This solution will be incubated with gentle shaking for two hours. The undigested hyphal material will be removed by layering the protoplasting mixture onto a 1.2 M sucrose cushion and centrifuging in

a clinical centrifuge. Protoplasts will be collected from the top of the sucrose cushion and placed in a centrifuge tube with equal volume 0.6 M KCl for centrifugation. The protoplasts will form a loose pellet, which will be resuspended in 2 mL of 0.6 M KCl and transferred to two new microcentrifuge tubes. Protoplasts will again be pelletized and resuspended in 0.6 M KCl 3 additional times, then the pellets will finally be resuspended in 0.5 mL of 0.6 M KCl and 50 mM CaCl2. Throughout the steps of this procedure, samples will be viewed under a microscope to record progress of protoplast production. (Szewczyk, 2006)

### *Transformation*

To begin the transformation, 10 μL of the constructed CRISPR-Cas9 vector will be added to 100 μL protoplast suspension and mixed thoroughly. 50 μL of PEG solution will be added and the solution will be vortexed and placed in an ice bath for 25 minutes. 1 mL of PEG solution will be added, mixed, and put in an ice bath for 25 minutes. 100 μL will be added to Fusarium complete media containing hygromycin. Resulting colonies will be isolated and streaked on hygromycin plates and left to grow as well (Szewczyk, 2006).

## Conclusion

Due to the clear phenotypic results of the growth of yellow colonies, it is concluded that the mutation of the yA gene in *Aspergillus nidulans* was successful. This initial step provided a useful introduction to the use of a CRISPR-Cas9 system for genetic editing in filamentous fungi. Familiarity with this system was essential for the transition to use in *Fusarium verticillioides,* a fungus that has frequented far fewer studies than *A. nidulans*. The primary use of the CRISPR-Cas9 system in *A. nidulans* greatly supported the construction of the CRISPR-Cas9 vector

specific for the FUM1 gene in *F. verticillioides* and the protocols to be followed in the collection of spores, production of protoplasts, and eventual transformation.

It is concluded that a successful construction of a target-specific CRISPR-Cas9 vector occurred. This is based on the results of the final PCR, showing the presence of both initial PCR fragments containing the guide RNA in the transformed colonies of *E. coli.* This isolated plasmid has been stored at -40℃ with the intentions of being used in the future PEG-mediated transformation of *Fusarium verticillioides* protoplasts. While it has been concluded that the intended plasmid has been constructed, it cannot be concluded whether this vector is successful in the mutation of the FUM1 gene without the introduction of the plasmid to the fungal pathogen and analysis of the corresponding results.

It is anticipated that in the event of a successful transformation of *F. verticillioides* protoplasts with this target-specific CRISPR-Cas9 vector, the FUM1 gene would be silenced. This could be detected with the use of hygromycin resistance as a selection marker. Since the constructed vector consists of a gene for hygromycin resistance, colonies could be grown and selected from plates containing hygromycin. The successful growth on these plates would support the successful transformation of this vector, and colony PCR could be performed on these colonies to amplify and eventually sequence the final product. This final sequencing information would give insight as to if the FUM1 gene had been disrupted by comparing it with the sequence of the gene in a wild-type colony that had not been transformed.

Strains of *Fusarium verticillioides* with a disrupted FUM1 gene fail to produce detectable levels of fumonisins, a known carcinogen (Alberts et al., 2017). The silencing of the FUM1 gene would provide colonies of *Fusarium verticillioides* that are potentially less pathogenic. These colonies could be grown and studied to further conclude whether or not the FUM1 gene had been

disrupted, the importance of FUM1 in pathogenicity, and perhaps offer insight into combating the pathogenicity of *Fusarium verticillioides.* Having a better understanding for what is causing pathogenicity allows scientists to better target and treat crops to protect against this species. This can lead to economic and human health benefits, making it an important research concern.

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