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Implementing CRISPR-Cas9 gene editing system in Microbotryum.

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IMPLEMENTING CRISPR/CAS9 GENE EDITING SYSTEM IN *MICROBOTRYUM*

By

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Abstract

Microbotryum dianthorum is a species of smut fungus which causes infection in plants in the *Dianthus* genus. The infection process is aided by small-secreted proteins known as effectors. Effectors may aid in the infection process because they are secreted by the fungus and can enter plant tissue, possibly to facilitate infection and/or manipulate the host. Thus, it is of interest to investigate the genes for these effectors to determine their role in the infection process. One method to study genes is through gene disruption, via the CRISPR/Cas9 gene editing system, but this has not yet been implemented in *Microbotryum dianthorum*. The goal of this study was to establish reliable CRISPR/Cas9 gene editing in *Microbotryum* species, so in the future it can be used to target genes of putative effectors. A vector containing *cas9* was constructed and was successfully used to transform *Microbotryum dianthorum.* The vector was designed to allow controlled expression of *cas9,* such that expression should be high with rich media and much reduced with low-nutrient media. This prediction was evaluated by measuring transcription levels using Quantitative Real-time Polymerase Chain Reaction (qRT-PCR). It did not appear, from qRT-PCR, that expression was regulated by nutrient levels as previously expected. Nevertheless, the data did confirm that the *cas9* gene was expressed in at least one *Microbotryum* species, paving the way for implementation of CRISPR/Cas9 targeted gene disruptions in this group of fungi.

Introduction

Microbotryum dianthorum is a smut fungus species complex (Kemler, Göker, Oberwinkler, & Begerow, 2006) that infects plants in the family *Caryophyllaceae,* also known as "pinks" or carnations. A majority of this complex generally infects species in the wildflower genus, *Dianthus,* although some of the species are specialists and thus only infect one species in the genus (Bucheli, Gautschi, Shykoff, & Shykoff, 2000). This is rather different from well-studied smut fungi like *Ustilago maydis* or other *Microbotryum* species that specifically infect plants of one particular species. *M. dianthorum* teliospores land on the plant, typically through insect pollination, germinate to produce cells capable of mating, produce infectious structures to enter the plant, and then the fungus overwinters in the plant. When the plant reemerges in the next growing season, its anthers (normally present in male flowers) display the fungus' teliospores instead of its own pollen (Schäfer, Kemler, Bauer, & Begerow, 2010). This infection process is thought to be aided by small-secreted proteins (SSPs) (Kuppireddy et al., 2017).

In a study of another *Microbotryum* species, *M. lychnidis-dioicae,* over half of the SSPs were shown to be significantly upregulated during infection, which suggests these proteins may aid in the infection process (Perlin et al., 2015). The proposed mechanism for this is that the fungus would invade plant tissue and release SSPs that would manipulate the plant's cellular processes and cause physiological changes that result in replacement of pollen in the anthers with fungal spores. The work on effectors was expanded upon by Kuppireddy et al. (2017), who identified and analyzed effectors in the infection process. In that work, potential host targets for several putative fungal effectors were identified in *M. lychnidis-dioicae*. This further highlighted the importance of the previous findings and suggested a need for more research into these putative effects. Thus, if CRISPR/Cas9 gene editing is effectively implemented in

Microbotryum, it could be used to disrupt the genes of putative SSPs in order to evaluate the impact on subsequent infection in *Microbotryum*.

CRISPR/Cas9 was first used as a gene editing method in 2012 when researchers realized different guide RNAs can be used with Cas9 to make cuts in DNA for gene editing (Jinek et al., 2012). CRISPR/Cas9 has already been used in a variety of organisms (Lone, Kama, Ahmad, Shahi, & Pokharel, 2018) and was used in the fungus *Saccharomyces cerevisi*ae for the first time in 2013 (DiCarlo et al., 2013). This opened up the opportunity to implement the CRISPR/Cas9 system in other species of fungi. Schuster, Schweizer, Reissmann, and Kahmann (2016) later established this system in the pathogenic smut fungus, *Ustilago maydis.* Species in both the genera *Ustilago* and *Microbotryum* are smut fungi, and the genera are similar enough that some *Microbotryum* species were classified as *Ustilago* species until Deml and Oberwinkler (1982) reclassified these fungi. Thus, it is not unreasonable to attempt to establish the CRISPR/Cas9 gene editing system in *Microbotryum dianthorum.* If CRISPR/Cas9 gene editing were successfully implemented, this system could be used to investigate the effects of small-secreted proteins (SSPs), which often play a role in plant infection. The goal of this study was to develop a CRISPR/Cas9 system for *M. dianthorum* species, one that might later be applicable to other species in the *Microbotryum* fungal complex. The overall strategy was to first produce strains of the fungus that express the *cas9* gene, preferably under the control of a regulatable promoter.

Materials and Methods

Plasmid Construction

A plasmid was constructed using Gibson Assembly (Gibson et al., 2010; Gibson et al., 2009) to act as a vector to introduce selected genes into the *Microbotryum* species in such a way that expression of those genes could be controlled. Restriction digestion opens an existing plasmid, allowing the insertion of a gene of interest and a promoter. The fragments to be cloned are first amplified using the Polymerase Chain Reaction (PCR). Overlapping primers were used so that the pieces (i.e., the desired promoter and the gene) could be ligated into the plasmid, which was first made linear by restriction enzyme digestion. The process of Gibson Assembly is depicted in Figure 1.

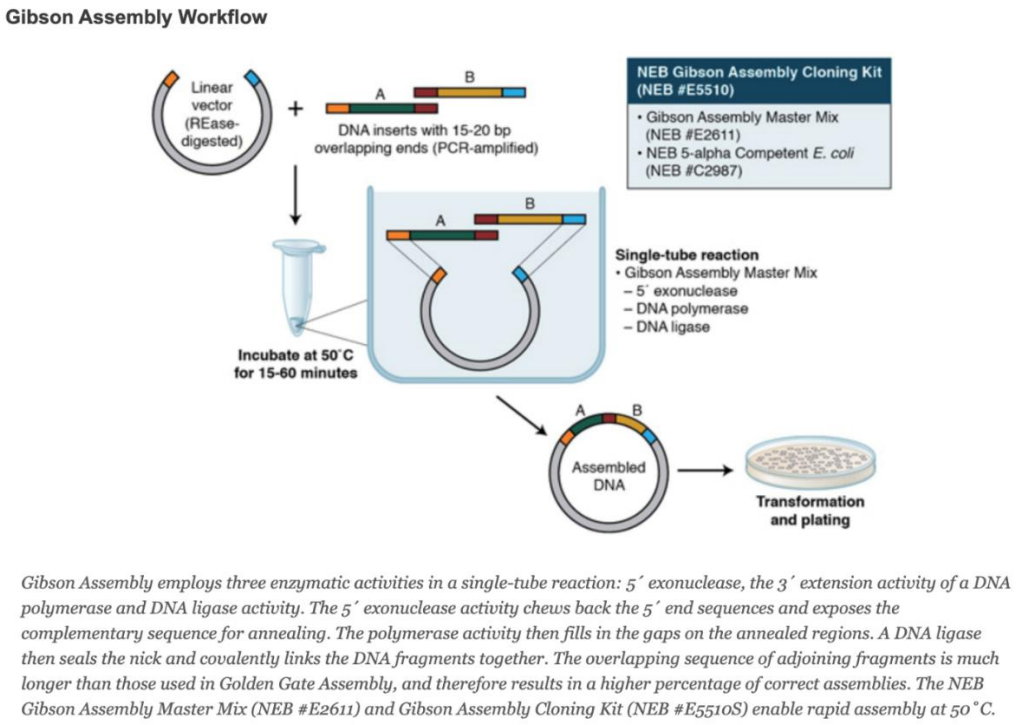

Figure 1. A schematic representation of Gibson protocol (Gibson et al., 2010; Gibson et al., 2009).

The initial plasmid, pMvHyg (See Appendix I; Toh, Treves, Barati, & Perlin, 2016), contained a kanamycin resistance gene and a hygromycin resistance gene to allow for selection of *E. coli* and *Microbotryum* cells, respectively. An inducible promoter, 5703P, and the *cas9* gene were added to pMvHyg using Gibson overlap and a set of primers (see Table 1) that bridged the gap between the promoter and the *cas9* gene, as well as ligating them to the ends of pMvHyg created by restriction digestion. The pieces to be used were amplified from source DNAs using PrimeStar Max high-fidelity DNA polymerase because the Gibson reaction requires DNAs that lack the A nucleotides added to PCR products by Taq DNA polymerase. The reactions joining these pieces were all done in a "one-pot" reaction, according to the procedure supplied by the manufacturer of a commercially-available kit (NEBuilder® HiFi DNA Assembly, New England BioLabs, Ipswich, MA). The resultant vector is pMvHyg5703Cas9.

Table 1. List of PCR primers used.

Primer Name	Sequence
5703CasL	5'-ACCTTCACGACACATATCCATGCCGCCTAAGAAG-3'
Cas5703R	5'-CTTCTTAGGCGGCATATGTGTCGTGAAGGTGTAGGG-3'
PacI5703L	5'-TTACGAATTCTTAATTAAAAAAACGCAGCAACACATTTC-3'
CasXbaIR	5'-TCTGCAGGTCGACTCTAGAGTAAAACGACGGCCAGTGAA-3'
HygFSwathi	5'-AAAAGTTCGACAGCGTCTCC-3'
HygRSwathi	5'-ATTTGTGTACGCCCGACAGT-3'
btubF	5'-CGGACACCGTTGTCGAGCCT-3'
btubR	5'-TGAGGTCGCCGTGAGTCGGT-3'
MVLG5703 qRTL	5'-AATGCCTTCTTTCTGGCGTA-3'
MVLG5703 qRTR	5'-ATAGGTCAGGCACGCTTGTT-3'

The *cas9* gene and 5703P promoter fragments were amplified using PrimeStar protocol for amplification of DNA. The CutSmart protocol (New England Biolabs) was used to perform a

restriction digestion of pMvHyg using the XbaI and PacI enzymes. The restriction digestion was run at 37 ℃ for 6 hours. DNA fragments were separated by agarose gel electrophoresis, and the bands of interested were isolated from gel slices using the Zymo Gel Purification kit. The plasmid, pMvHyg5703Cas9, constructed from Gibson overlap of the relevant pieces, i.e., the hygromycin resistance gene, the 5703P inducible promoter, and *cas9*, was sent for DNA sequencing (Eurofins, Louisville, KY) to confirm the presence of all parts of the construct and proper orientation of the genes and promoter (see Fig. 2).

The inducible promoter is important for testing the efficacy of Cas9 once the vector has been introduced into *Microbotryum*. The expression of genes associated with the inducible promoter should vary depending on which media the organism containing the promoter and the genes it controls is grown. When the organism is grown on yeast extract peptone dextrose (YPD), there should be high expression of *cas9* because the promoter upregulates transcription of the gene under conditions of high nutrient content. When the organism is grown on potato dextrose agar (PDA), there should be low expression of *cas9* because the promoter downregulates transcription of the gene under conditions of low nutrient content. Control of *cas9* expression is important since it has been shown that Cas9 can introduce unintended changes in genomes if expressed constitutively (Zhang, Tee, Wang, Huang, & Yang, 2015).

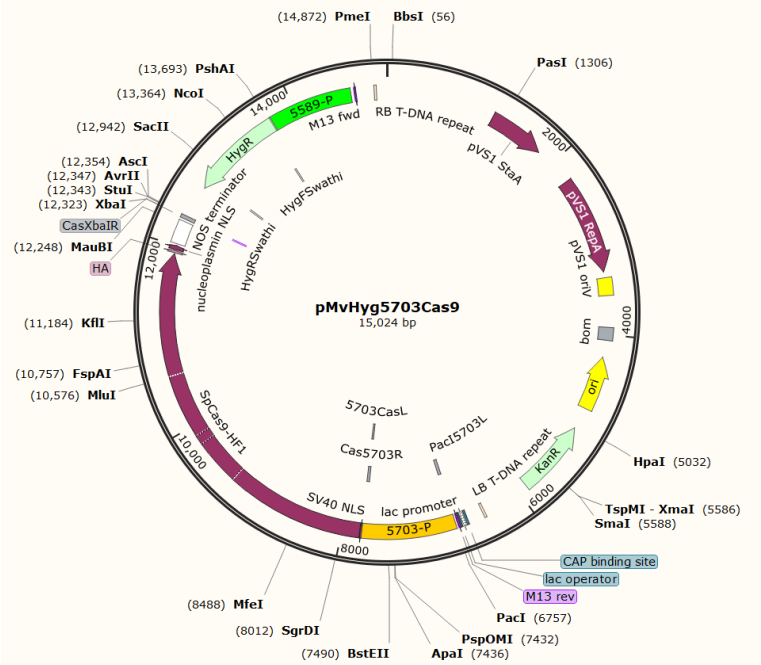

Figure 2. Plasmid map of pMvHyg5703Cas9 constructed via Gibson assembly. The plasmid map was produced in SnapGene (https://www.snapgene.com/). The plasmid is derived from plasmid pMvHyg (Appendix I; Toh, Treves, Barati, & Perlin, 2016), and as such, contains a kanamycin resistance gene (KanR), for selection of bacterial cells, a hygromycin resistance gene (HygR), for selection of *Microbotryum* cells, and Right (RB) and Left (LB) borders for transmission of the intervening sequences to *Microbotryum* via *Agrobacterium*-mediated transformation. The *hyg* gene was amplified during PCR using the HygFSwathi and HygRSwathi primers as denoted on the plasmid map (Fig. 2) and yielded an expected band of 877 bp (Fig. 5). Additionally, in this derivative, the high-fidelity version of the *cas9* gene (a generous gift from G. Doehlmann) was cloned, along with the 5703P promoter, between the XbaI and PacI sites, via Gibson overlap assembly (See Materials and Methods text). The *cas9* gene was amplified by PCR using 5703CasL and CasXbaIR primers as denoted on the plasmid map and yielded an expected band of 4.5 kbp (Fig. 3, Fig 4, Fig 6).

Transformation of *E. coli* to select for cells containing the *cas9* construct

Once the vector had successfully been constructed, it needed to be introduced into *Escherichia coli*, which was accomplished with heat shock transformation. RbCl-competent 10× DH5α cells were used. First, competent cells were thawed on ice. RFII buffer and then the vector DNA were added to the tube. The tube was incubated on ice for 30 min before incubating at 42 ℃ for 45 s. The cells were then incubated on ice for 2 min. This process creates holes in the membrane of the *E. coli* through which the DNA can enter the cell. The treated *E. coli* cells were then plated on Luria-Bertani (LB) agar containing 50 μg/mL kanamycin. The kanamycin in the agar will allow selection for only *E. coli* cells containing the construct because only transformed *E. coli* will harbor the kanamycin resistance gene. Colonies grew on the LB kanamycin plates after one day. Cells were scraped from the colonies and replated on to another LB plate and grown overnight at 37 ℃. These colonies were then collected and used to inoculate tubes of Circle Grow broth containing 50 μg/mL kanamycin. The tubes were incubated at 37 ℃ on a shaker overnight. Plasmid DNA was extracted using a purification that employs alkaline lysis, using the Wizard Kit (Promega) plasmid mini-prep protocol with slight modifications. The presence of the plasmid was confirmed by agarose gel electrophoresis; then the plasmid preparation was used in PCR reactions with primers (5703CasL and CasXbaIR; see Table 1) that flank the *cas9* gene to screen for the presence of the *cas9* gene using gel electrophoresis. The agarose gel was run at 70 V for about an hour. The standard PCR conditions with the ExTaq protocol were as follows. Initial denaturation was at 94 ℃ for 4 min. This was followed by 35 cycles of 94 ℃ for 30s, 60 ℃ for 30s, 72 ℃ for 5 min. A final extension was for 5 min at 72 ℃. Reactions were kept at 4 ℃ until use.

Transformation of *Agrobacterium* and *Microbotryum* strains

The confirmed construct next needed to be transformed into *Agrobacterium tumefaciens* strain EHA 105. *Agrobacterium tumefaciens* is a species of bacteria that uses horizontal gene transfer to infect plant cells and cause tumors in plants. The process by which *Agrobacterium* transfers DNA can be utilized in other organisms, such as *Microbotryum*, by transferring the construct from *E. coli* to *Agrobacterium* to *Microbotryum*.

A. tumefaciens competent cells were transformed with pMvHyg5703Cas9 by electroporation. In order to transform *Agrobacterium*, competent *Agrobacterium* cells and the DNA were transferred to chilled electroporation cuvettes and placed inside the Gene Pulser machine. The Gene Pulser (Bio-Rad) was set to a voltage of 2.5 kV, a "CAP" setting of 25 μFD, the Pulse Controller Unit to 400 Ω, and the time to 9 msec. Then, 1 mL of growth medium was added to the cuvettes directly after electroporation and the cells were placed on ice. The transformed cells were incubated at 28 ℃ for about 2 h before being plated onto LB plates containing 50 μg/mL kanamycin and 150 μg/mL spectinomycin. *Agrobacterium tumefaciens* (EHA105) requires the LB kanamycin spectinomycin medium because the strain utilizes a binary plasmid system. The plates were incubated at 28 ℃ for two days before replating colonies for screening and storage. Successful *A. tumefaciens* transformants (kanamycin- and spectinomycin-resistant) were again confirmed by PCR amplification of the *cas9* gene from plasmid DNA of selected kanamycin-resistant *Agrobacterium* colonies and screening the products via agarose gel electrophoresis. The DreamTaq Hot Start Master Mix was used along with left and right primers for *cas9* (5703CasL and CasXbaIR, respectively; see Table 1). DNA from 6 colonies of transformed *Agrobacterium* and pMvCRISPR, Proto 3, and pMvHyg DNAs (see Appendix I for plasmid maps) as controls, were used in the PCR. The pMvCRISPR and Proto 3 plasmids were

both used as positive controls because pMvCRISPR and Proto 3 are both plasmids containing the *cas9* DNA being screened for in the *Agrobacterium.* pMvHyg was intended as a negative control because it is a plasmid that should not allow amplification of the target gene(s) by the provided primers (see Table 1). The agarose gel was run at 70 V for about an hour.

Once the *A. tumefaciens* strain(s) bearing the correct construct were obtained, transformation of *Microbotryum* strains could ensue. Cultures of three different *Microbotryum* species were grown for transformation. The species grown were *Dianthus pavonius* MvDp 6, *Dianthus pavonius* 2.94, and, as a control, *Microbotryum lychnidis-dioicae* P1A. *Microbotryum lychnidis-dioicae* P1A strain was used since it had been successfully transformed earlier using the *Agrobacterium*-mediated approach (Toh et al. 2016). Each of these species has two mating types, A1 and A2; however, for these experiments only the A1 strain of each species of *Microbotryum* was used: MvDp 6D, P1A1, and 2.94D.

The *Microbotryum* transformation followed the previously established protocol for *Agrobacterium*-mediated transformation of *Microbotryum lychnidis-dioicae* (Toh, Treves, Barati, & Perlin, 2016). *Agrobacterium* strains that contained the construct were plated and allowed to grow at 28 ℃ for 24 h prior to setup. One mating type of each strain of *Microbotryum* was also incubated at 28 ℃ for four days prior to transformation. After the transformation, the *Microbotryum* cells were plated onto YPD with 150 μg/mL hygromycin and 300 μg/mL cefotaxime at 28 ℃ until colonies appeared. The hygromycin was used to select for transformed *Microbotryum* cells, while the cefotaxime was used to select against remaining *A. tumefaciens* cells. Colonies appeared after 16 d and were then purified by re-streaking onto YPD hygromycin cefotaxime plates.

DNA extraction and PCR

Total DNA from transformed *Microbotryum* cells was obtained using a protocol that lyses the cells and treats the extract with phenol:chloroform:isoamyl alcohol to remove proteins and polysaccharides (Toh et al., 2016). DNA in the aqueous layer was then precipitated with ethanol. The extracted DNA was used for PCR to amplify the hygromycin resistance gene and, separately, the *cas9* gene, and the results were then analyzed via agarose gel electrophoresis. Amplification of the hygromycin resistance gene fragment used left and right primers, HygF and HygR Swathi, respectively (see Table 1), while amplification of the *cas9* fragment used left and right primers, 5703CasL and CasXbaIR, respectively (see Table 1). The DreamTaq Hot Start PCR master mix was used as described previously for ExTaq reactions. The PCR products were separated in agarose via electrophoresis and compared against PCR reactions using plasmids pMvHyg and pMvCbx5589, and H_2O as a "no DNA" negative control. The pMvHyg acted as a positive control for the hygromycin fragment, while pMvCbx5589 does not contain the hygromycin resistance gene and thus, should not be amplified by the primers used, i.e., a negative control. Reactions screening for *cas9* were compared against PCR reactions using plasmids pMvHyg5703Cas9 and pMvHyg, and separately, H_2O instead of added DNA.. The pMvHyg5703Cas9 plasmid contains *cas9* so it acted as a positive control, while pMvHyg does not contain the *cas9* and thus, should not be amplified by the primers used, i.e., a negative control. Water was also used as a negative control to test for any contamination present in the primers or master mix. The gel was run at 70 V for about an hour.

RNA Extractions and Real-Time qRT-PCR

Once it had been confirmed that the construct DNA was present in *Microbotryum*, tests were needed to determine if the *cas9* expression occurred and whether such expression was different, as expected, under different growth conditions. To do this, RNA extractions were performed using the Zymoclean kit, and were used to screen for *cas9* transcription via Real-Time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR).

First the *Microbotryum* transformant cells were plated on PDA, the low nutrient media condition that leads to downregulation of the *cas9* gene via the 5703P promoter, and also onto YPD, the high nutrient media condition that leads to upregulation of the *cas9* gene (from RNA-Seq data of the MVLG_05703 gene; Perlin et al., 2015). RNA extractions were conducted from a set of three media plates for each condition that were grown overnight at 28 ℃ to produce enough biomass for the extractions. The different conditions can be seen in Table 2. Cells were collected from the plates and the nucleic acids were isolated from the cells through manually crushing of the cells in liquid nitrogen, followed by extraction purification using the kit and eventually ethanol precipitation. The sample was then treated with DNase to break down the DNA present in the sample, leaving only RNA.

Table 2. Concentrations of RNA extracted from each *Microbotryum* strain, as determined by Nanodrop spectrophotometer, and condition. RNA concentrations were then used to determine the expected amount of cDNA produced.

Microbotryum strain and media	Concentration of RNA
MvDp 6D wild type grown on YPD	759.1 ng/µL
MvDp 6D wild type grown on PDA	821.9 ng/µL
MvDp 6D x pMvHyg5703Cas9 grown on YPD	1358.5 ng/µL
MvDp 6D x pMvHyg5703Cas9 grown on PDA	1800.3 ng/µL
P1A1 x 5589Cas9 grown on PDA (positive control)	492.5 ng/µL
P1A1 x 5589Cas9 grown on YPD (positive control)	766.4 ng/µL
P1A1 wild type grown on YPD	652.3 ng/µL
P1A1 wild type grown on PDA	1559.4 ng/µL
P1A1 x pMvHyg5703Cas9 grown on YPD	1395.7 ng/µL
P1A1 x pMvHyg5703Cas9 grown on PDA	1551.4 ng/µL

The amount of RNA produced for each sample was determined using a Nanodrop instrument. cDNA was synthesized from the extracted RNA by combining the dried RNA template with 6.5 µL of water and mixing with 0.5 µL of the Oligo dT primer provided in the cDNA synthesis kit, and 0.5 µL of deoxynucleotide triphosphates (dNTPs). This mixture was incubated at 65 ℃ for 5 min and then placed on ice for 2 min. An enzyme mix of 2 µL 5X buffer, 0.5 µL 100 mM DDT, 0.5 µL RNase OUT, and 0.5 µL Superscript IV reverse transcriptase was added to the RNA mix. This was then incubated at 50 ℃ for 10 min followed by 80 ℃ for 10 min. This process produced cDNA for use in Real-Time qRT-PCR.

The reaction mix for qRT-PCR includes EvaGreen dye (Mango Biotech), left and right primers for *cas9* (see Table 1), and the cDNA that was generated from the RNA extractions. EvaGreen dye binds to dsDNA and fluoresces upon binding, allowing visualization of the

presence and thus amplification of the target *cas9* cDNA. The first qRT-PCR conducted tested the expression of the *cas9* gene against the expression (using primers btubF and btubR; see Table 1) of the β-tubulin gene, *MVLG_00180,* which is a housekeeping gene and thus always expressed at the same level, regardless of nutrient conditions. A follow-up qRT-PCR was conducted to test again if *cas9* was differentially expressed depending on nutrient condition.

qRT-PCR was conducted again using the previously made cDNA from the MvDp 6D wild type and transformants, the P1A1 transformant that is a positive control for *cas9,* and the P1A1 wild type and transformant, to test if the endogenous *MVLG_05703* gene was being differentially expressed depending on media type. The reaction mix for qRT-PCR was the same as previously described except for the primers. Left and right primers (MVLG5703 qRTL and MVLG5703 qRTR, respectively; see Table 1) for the *MVLG_05703* gene were used instead, allowing for amplification of the target *MVLG_05703* cDNA.

Results

Plasmid Construction

Putative *E. coli* transformants were screened via PCR for presence of pMvHyg5703Cas9 (Fig. 3). Each of the samples (A8, B8, and C8) produced a band at about 4.5 kbp which was the expected size. No band was shown in the sample for the negative control (E8). A much smaller band of PCR amplified DNA was seen in all of the samples. This band was determined to be likely due to mis-priming by the Cas5703L primer, which will alone prime a 310 bp sequence in pMvHyg using the last 6 nucleotides of the primer. Thus, the band appeared in reactions from all of the transformants and the positive control plasmids, both of which are derivatives of pMvHyg (Fig 3).

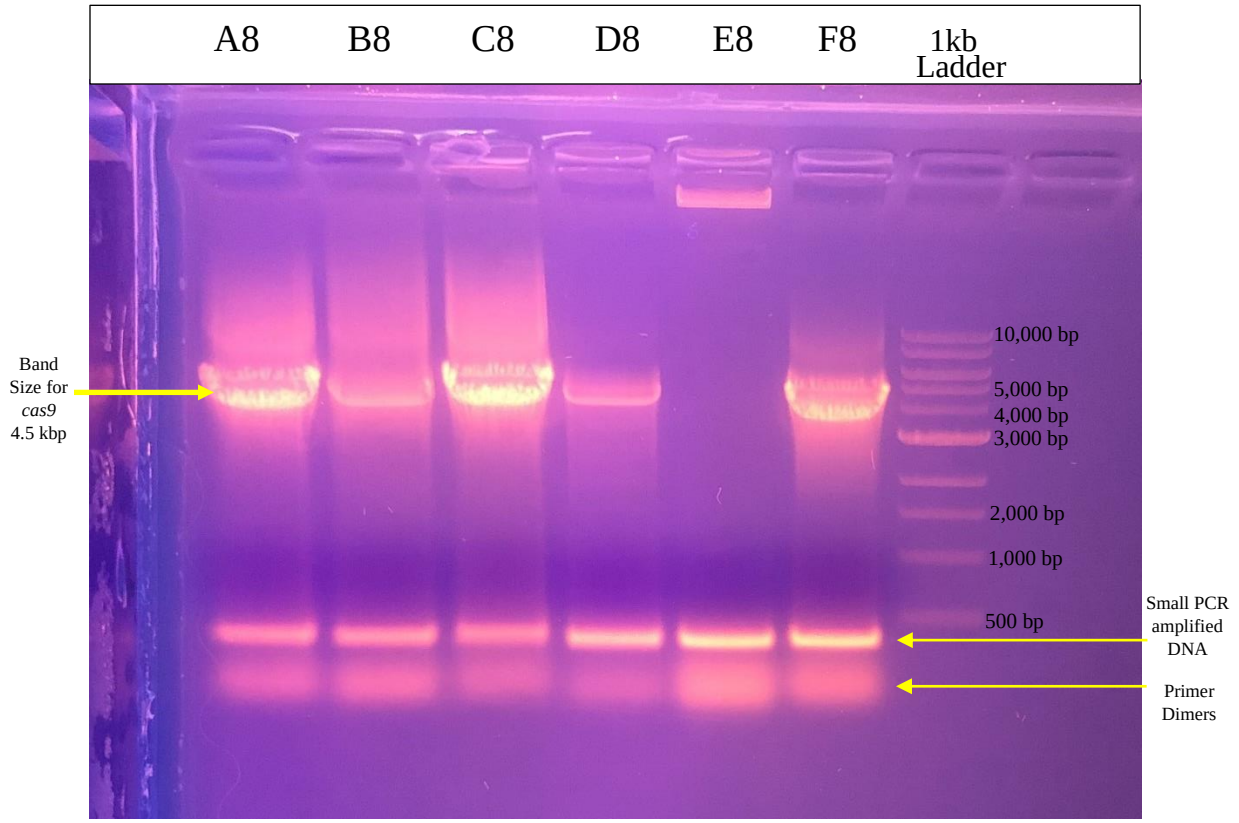

Figure 3. A gel electrophoresis gel run to screen for the presence of the *cas9* gene in *Escherichia coli* transformants. PCR reactions using primers that would amplify the *cas9* gene were used in all samples, which were then loaded into an agarose gel for separation via electrophoresis. Samples were loaded into the following lanes: A8) Hyg5703Cas9 vector #2; B8) Hyg5703Cas9 vector #8; C8) Hyg5703Cas9 vector #12; D8) CbxCas5703 vector #14; E8) pMvCbx (negative control); F8) plasmid #1782 (Source of *cas9*; positive control); 1 kb ladder (New England Biolabs), as size standard.

Transformation into *Agrobacterium* and *Microbotryum*

Six colonies of *A. tumefaciens* were collected from transformation plates. Of these, all six were screened via PCR for presence of the desired plasmid. The gel containing the PCR products (Fig. 4) showed promising results, with only one sample displaying a negative result. The two positive controls (pMvCRISPR and Proto 3) both showed a band at the expected 4.5 kbp band size for the *cas9* gene and the negative control (pMvHyg) showed no band at the expected band size for the *cas9* gene. This confirmed the presence of *cas9* in the transformed *A. tumefaciens*. Again, as seen in Fig. 3, a smaller PCR amplified DNA band was seen for all DNAs derived from the pMvHyg plasmid (Fig. 4).

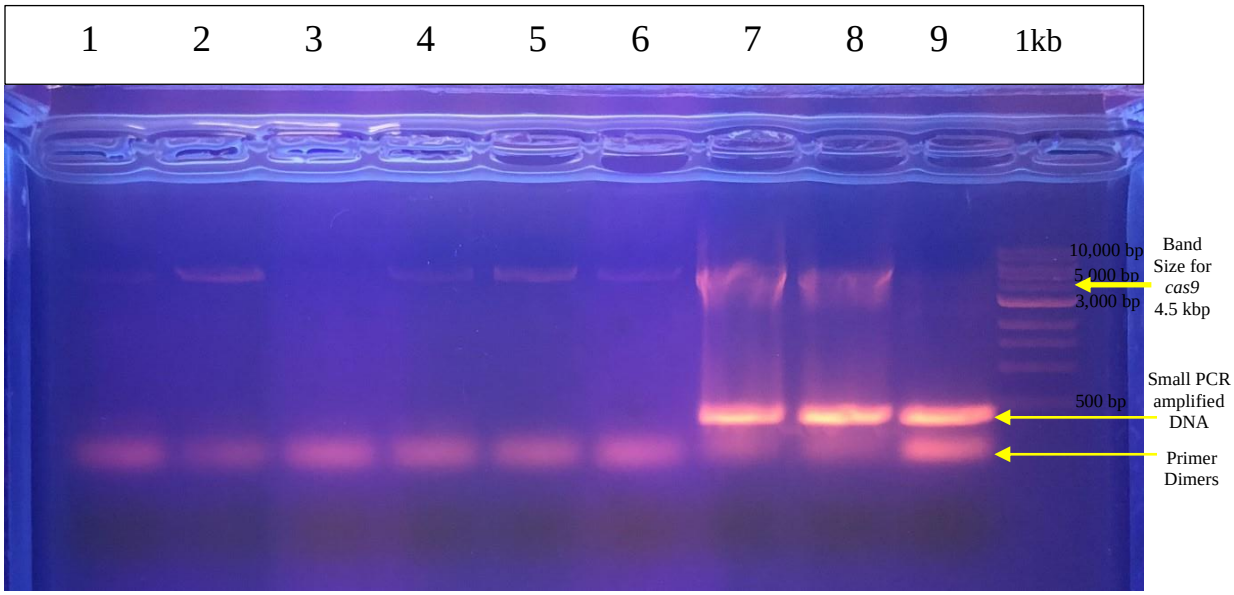

Figure 4. A gel electrophoresis gel run to screen for the presence of the *cas9* gene in *Agrobacterium tumefaciens* transformants. PCR reactions using primers that would amplify the *cas9* gene were used in all samples, which were then loaded into an agarose gel for separation via electrophoresis. Samples were loaded into the following lanes: 1) AgroHyg5703Cas9 1; 2) AgroHyg5703Cas9 2; 3) AgroHyg5703Cas9 3; 4) AgroHyg5703Cas9 4; 5) AgroHyg5703Cas9 5; 6) AgroHyg5703Cas9 6; 7) pMvCRISPR (positive control); 8) Proto 3 (positive control); 9) pMvHyg (negative control); 1 kb ladder (New England Biolabs), as size standard.

For the *Microbotryum* transformations, colonies appeared on the YPD hygromycin cefotaxime plates after 16 days. Colonies appeared for the MvDp 6D (*Dianthus pavonius)* and the P1A1 (*Microbotryum lychnidis-dioicae)* strains but not for the 2.94D (*Dianthus pavonius)* strain. Over 20 colonies were obtained for MvDp 6D transformation and 4 appeared for P1A1 transformation. The colonies were replated on YPD hygromycin cefotaxime plates and left to grow for two days at 28 ℃.

The agarose gel of the PCR amplified DNA from *Microbotryum* transformants (Fig. 5) showed the presence of pMvHyg5703Cas9 because there was a band of DNA at the expected size (877 bp) for the *hyg* gene, which can also be seen in the positive control. The negative control, pMvCbx5589, produced a faint band but this was determined to be a small amount of contamination or a loading error because no mis-priming by the primers used was identified that would cause a band of this size. Water, in place of DNA, was used as another negative control and produced no band where one would be expected if the sample contained the *hyg* DNA, so it was concluded that the transformed *Microbotryum* did in fact contain the pMvHyg DNA inserted into the genome (Fig. 5).

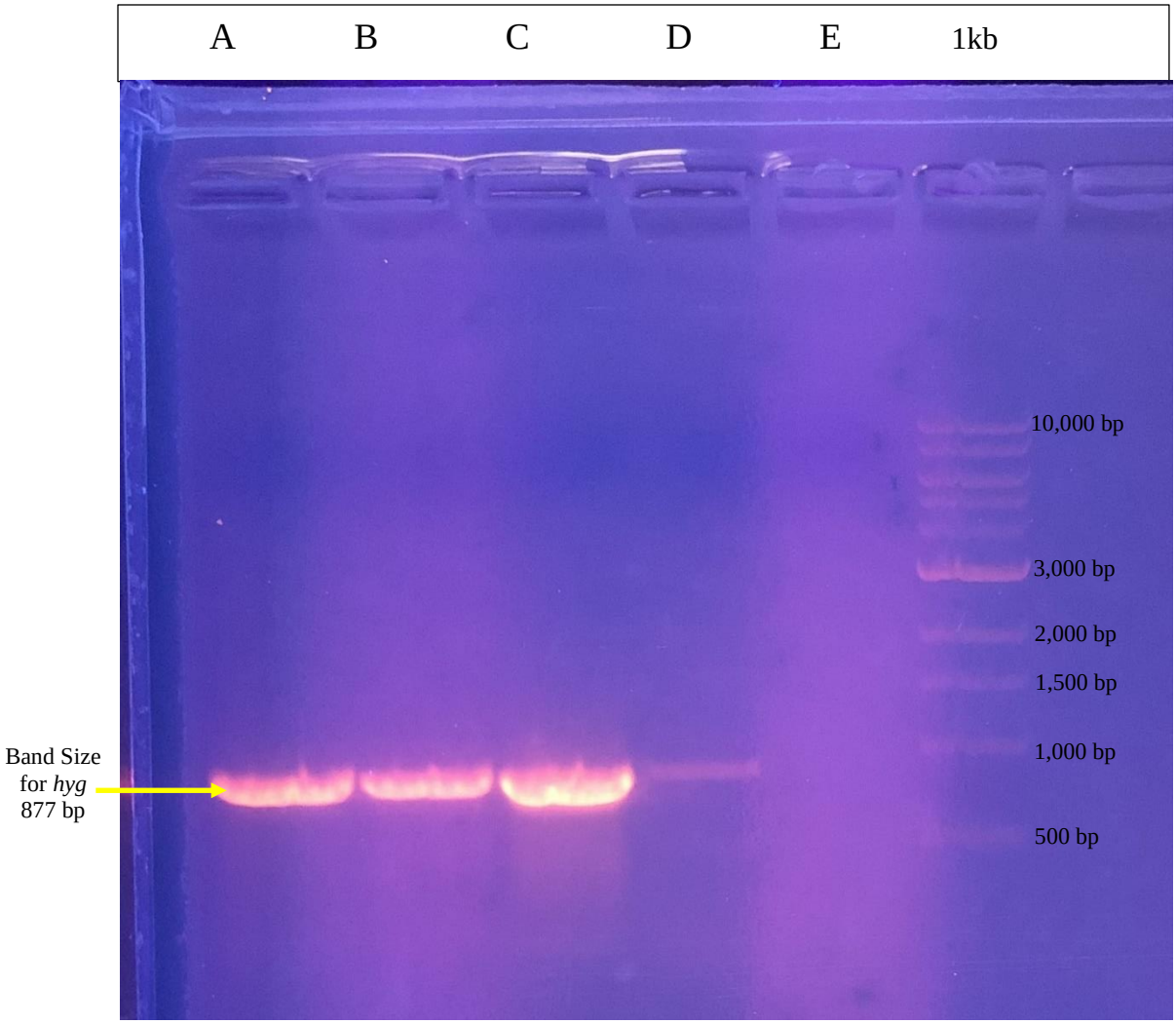

Figure 5. Agarose gel electrophoresis used to screen for the presence of *hyg* in *Microbotryum* transformants. PCR reactions using primers that would amplify the *hyg* gene were used in all samples, which were then loaded into an agarose gel for separation via electrophoresis. Samples were loaded into the following lanes: A) MvDp 6D x AgroHyg5703Cas9 A1; B) MvDp 6D x AgroHyg5703Cas9 A2; C) pMvHyg (positive control); D) pMvCbx5589 (negative control); E) H_2O (negative control); 1 kb ladder (New England Biolabs), as size standard.

The agarose gel of the PCR amplified DNA from *Microbotryum* transformants (Fig. 6) showed the presence of pMvHyg5703Cas9, as evidenced by a band of DNA at the expected size

(4.5 kbp) for the *cas9* gene. The positive control, pMvHyg5703Cas9, also showed a band at the expected 4.5 kbp band size for the *cas9* gene, while the negative control (pMvHyg) produced no band at the expected band size for the *cas9* gene. The positive control showed a smaller PCR amplified DNA band as seen in Fig. 3 and Fig. 4 because the construct was derived from pMvHyg. The pMvHyg sample (negative control) also produced a smaller PCR amplified DNA band around 1 kbp but no additional mis-priming was identified that would account for this band. Water, in place of DNA, was used as another negative control and produced no band where one would be expected if the sample contained *cas9.* This confirmed that the transformed *Microbotryum* did, in fact, contain the pMvHyg5703Cas9 DNA in the genome (Fig 6).

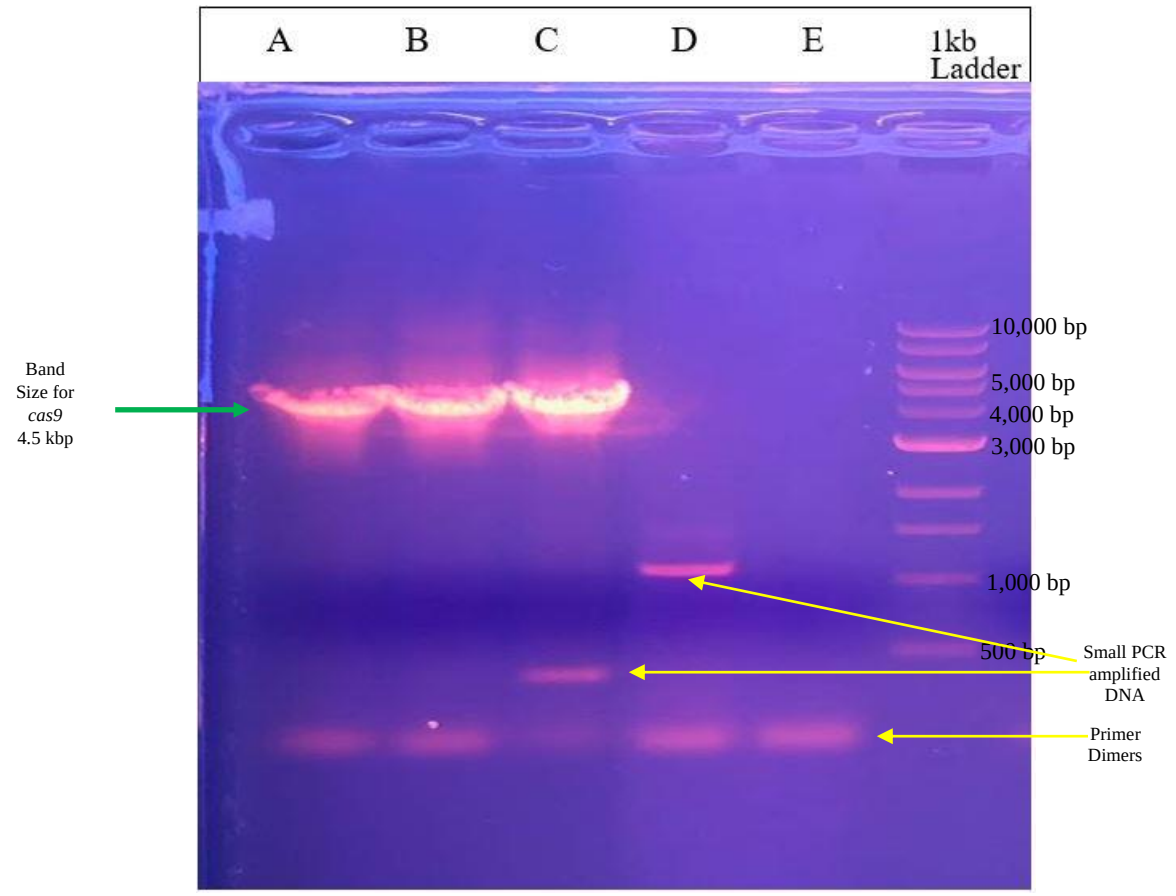

Figure 6. Agarose gel electrophoresis used to screen for the presence of *cas9* in *Microbotryum* transformants. PCR reactions using primers that would amplify the *cas9* gene were used in all samples, which were then loaded into an agarose gel for separation via electrophoresis. Samples were loaded into the following lanes: A) MvDp 6D x AgroHyg5703Cas9 A1; B) MvDp 6D x AgroHyg5703Cas9 A2; C) AgroHyg5703Cas9 (positive control); D) pMvHyg (negative control); E) H_2O (negative control); 1 kb ladder (New England Biolabs), as size standard. Green arrow indicates location of expected sized band for *cas9* gene product.

Measurement of Gene Expression via Real-Time qRT-PCR

The Real-Time qRT-PCR was expected to show *Microbotryum* transformants grown on the low nutrient PDA agar would display no or very low expression of the *cas9* gene and the *Microbotryum* transformants grown on the high nutrient YPD would display high expression of the gene. This outcome was not observed (Fig. 7). Instead, the *cas9* gene was expressed on both media at similar levels, indicating that the 5703P promoter does not control expression of *cas9* in an inducible fashion, as was expected based on prior experiments. Samples were tested against an endogenous control using primers for the β-tubulin gene, *MVLG_00180*, to normalize for comparison of expression levels between *cas9* and the β-tubulin gene. No distinct difference in relative expression of the *cas9* gene was seen on different media types. However, since only a single set of biological samples (i.e., only one sample of cDNA was produced from a single RNA extraction for each sample) were used here, statistical analyses could not be used for determination of standard error and log2 fold changes between conditions (Fig. 7).

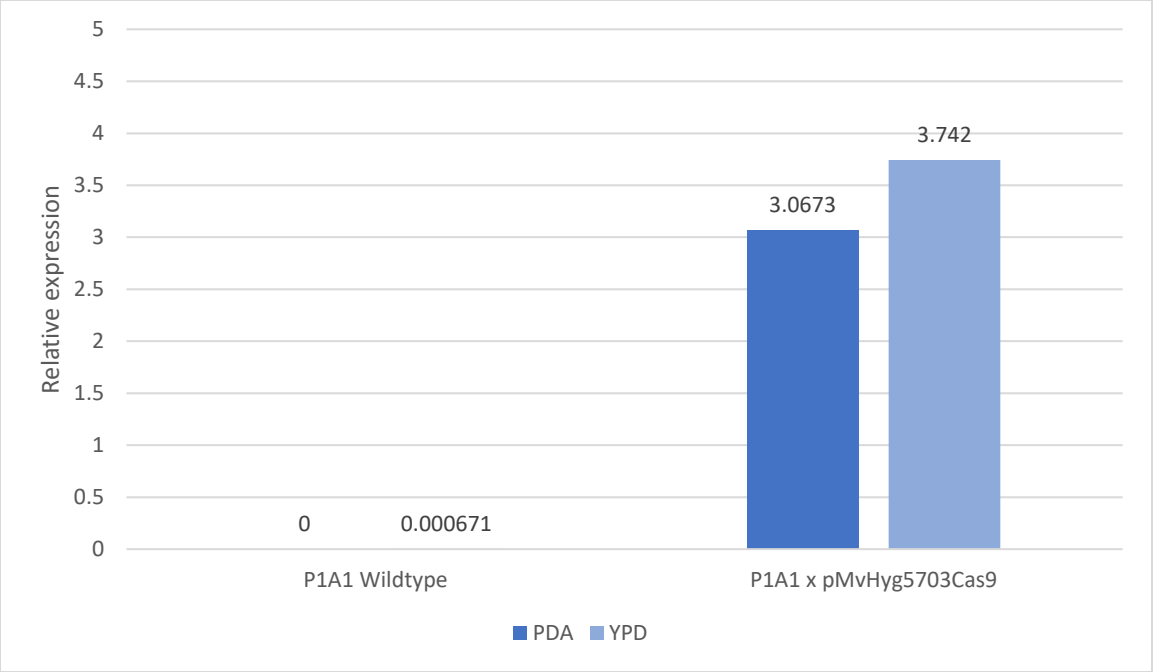

Figure 7. qRT-PCR of *cas9* expression. Samples grown on PDA are shown in dark blue and samples grown on YPD are shown in light blue. Strains whose cDNAs were tested are indicated on the *x*-axis. RNAs used for this qRT-PCR were from only one biological sample each. Raw data can be viewed in Appendix II.

Another qRT-PCR was run to measure relative expression depending on media type (Fig. 8). Again, this test showed no clear difference in relative expression of *cas9* depending on media type. There also did not appear to be measurable levels of transcription of the *cas9* gene in the transformant of the MvDp 6D background, at least none above those negligible levels found in the untransformed wild type progenitor strain.

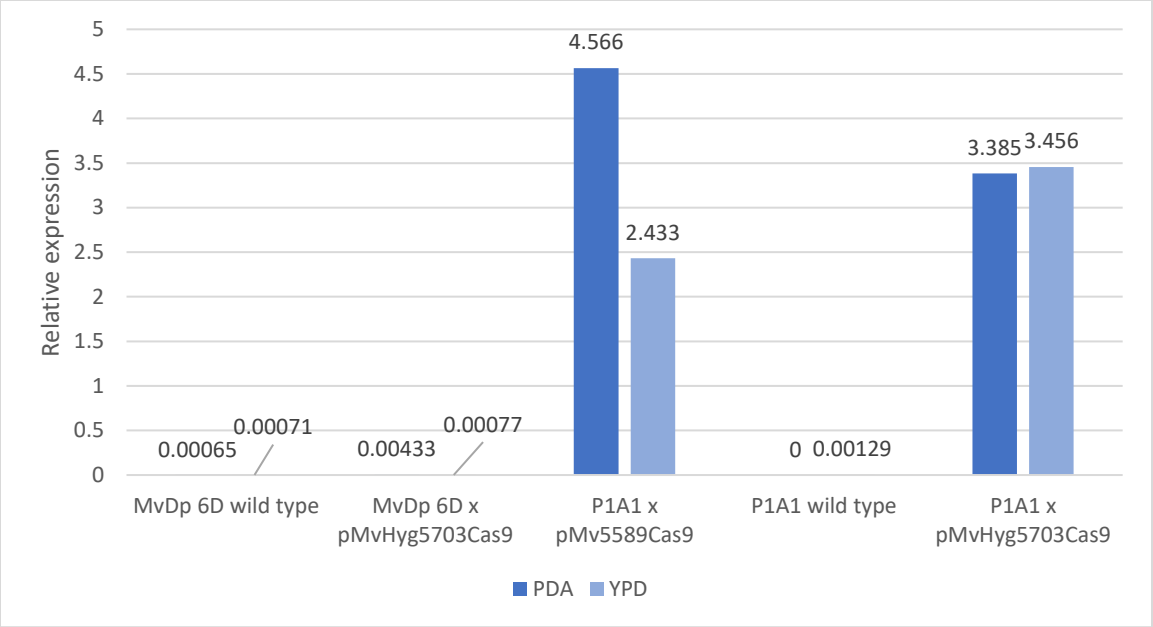

Figure 8. qRT-PCR of *cas9* expression. Samples grown on PDA are shown in dark blue and samples grown on YPD are shown in light blue. Strains whose cDNAs were tested are indicated on the *x*-axis. RNAs used for this qRT-PCR were from only one biological sample each. Raw data can be viewed in Appendix II.

This led to further qRT-PCR testing for the *MVLG_05703* structural gene to assess whether the 5703P promoter was modulating expression based on media condition at all (Fig. 9). Expression data was normalized against an averaged expression of the housekeeping β-tubulin gene *MVLG_00180.* The samples showed an appreciable difference in expression levels but not as expected, where expression would be higher on YPD media than PDA media, which further supports the finding that the 5703P promoter does not reliably alter expression of the *cas9* gene associated with this promoter in response to high and low nutrient levels, in contrast to expectation.

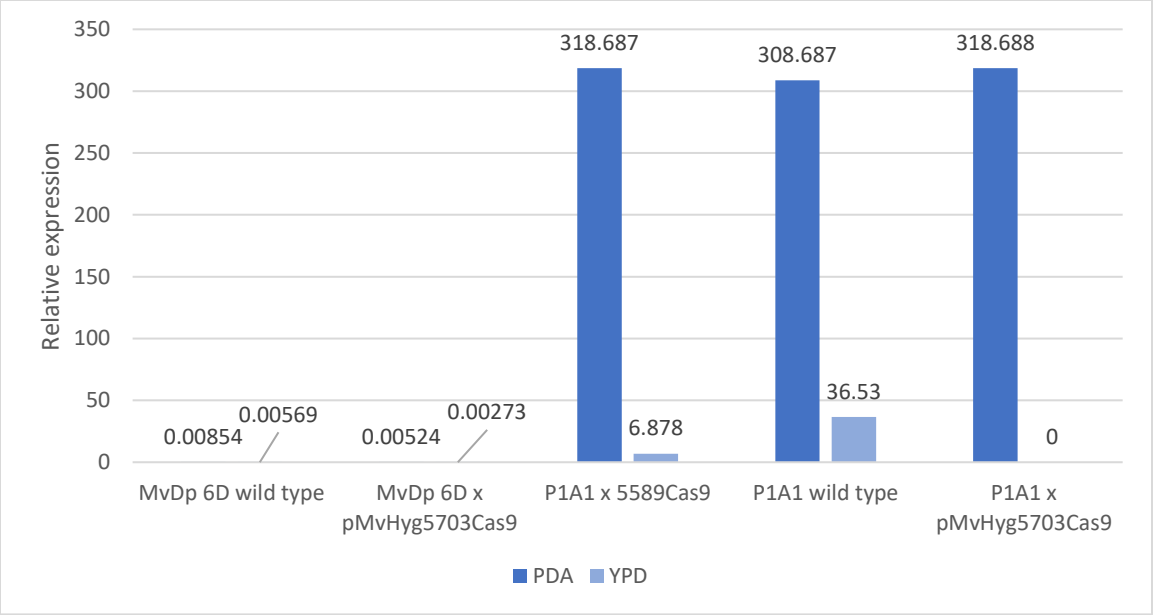

Figure 9. qRT-PCR of *MVLG_05703* expression. Samples grown on PDA are shown in dark blue and samples grown on YPD are shown in light blue. Strains whose cDNAs were tested are indicated on the *x*-axis. RNAs used for this qRT-PCR were from only one biological sample each. Raw data can be viewed in Appendix II.

Discussion

Through this research a reliable transformation system in *Dianthus pavonius* and thus, the *Microbotryum dianthorum* group of species, has been demonstrated. What has not been demonstrated, however, is that the expression of *cas9* can be modulated by the 5703P promoter. Also, although *cas9* transcription was shown in the P1A1 *cas9* transformants, no such expression has yet been observed in the MvDp 6D transformants. Since the MvDp strains also failed to show expression of the *MVLG_05703* endogenous gene in these studies, one possible explanation is that the RNA extracts/cDNAs for these isolates were somehow defective. To test this, the qRT-PCR tests need to be run again with more biological replicates and additional known MvDp genes should be tested for expression via qRT-PCR. Alternatively, for the *MVLG_05703* endogenous gene, it may be that the primers that work for P1A1 (an isolate of *M. lychnidis-dioicae* instead of *M. pavonius*) do not work for the latter species. One way to check that is to test if those primers can amplify *MVLG_05703* from genomic DNA extracted from these MvDp 6D strains. If the DNA yields a band with the 5703 primers, then there is no expression or the cDNA is poor quality. If the DNA does not yield a band, then those primers may not work for MvDp, possibly due to differences in that gene in MvDp versus in P1A1. A preliminary comparison of the *M. pavonius* genome (not yet fully annotated) showed a number of point mutations and insertions/deletions for the *MVLG_05703* orthologous region in *M. pavonius* (data not shown) and these are currently being examined for changes that would have affected PCR primer binding.

Cas9 has been shown to have unintended effects of the genome if expression is high (Zhang et al., 2015) thus, it was of interest to use an inducible promoter to regulate when Cas9 is expressed so it would only affect genes of interest. Prior to this research it was assumed that the

5703P promoter was an inducible promoter based on the nutrient content of the media on which the organism had been grown (Perlin et al., 2015); however, this research has demonstrated that it does not modulate expression of *MVLG_05703*, the native structural gene controlled by 5703P, in the expected fashion. In fact, the preliminary results presented here showed the opposite of expectation, at least for the P1A1 strain background (See Fig. 9). This shows that the promoter is likely not inducible based on media type. One caveat is that only one biological sample was used for each strain studied. Normally, a minimum of 3 biological replicates is necessary for statistical analyses of expression data such as qRT-PCR. Additional experiments will include more biological replicates for the analyses. A future direction to this project in particular depends on a working inducible promoter. It would be of interest to attempt this experiment again with a different inducible promoter that has promise in regulating Cas9 production, so as to prevent unnecessary Cas9 expression when not attempting to do a disruption or after a disruption has been made.

Identification of Target Genes for Disruption via Cas9

Since confirming the transformation of the pMvHyg5703Cas9 vector into *Microbotryum,* and the expression of the *cas9* gene using the inducible promoter, the next step is to pick an effector gene to target for CRISPR-Cas9-mediated disruption. Genes of interest would be determined through bioinformatics work that identifies putative small-secreted proteins. Once a gene of interest is determined, guide RNAs based on this effector gene sequence would be designed to target the disruption of the gene, allowing Cas9 the ability to target the gene of interest.

Plant Infections

Once the gene has been disrupted, plants would be infected with a wild type strain and a mutant strain. Considering transformant colonies were from the MvDp 6D (*Dianthus pavonius)* and P1A1 (*Microbotryum lychnidis-dioicae) Microbotryum* strains, a wild type and a transformant MvDp 6D and a wild type and a transformant P1A1 strain would likely be used for infection. Alternatively, if mutants from compatible mating-type strains are generated, it would be of interest to conduct infections in which both mating partners contain the mutation in the targeted gene. After infection, the plants would be closely monitored to determine phenotypic differences in infection progression.

If the effector were crucial to the infection process, potential differences would be the wild type infected plants progressing to disease more quickly than the transformant infected plants or the symptoms being more severe in the wild type afflicted plants. If there were no effect from disrupting the effector, wild type and transformant infected plants would have about the same severity of infection and same progression to a diseased state, including spore production in infected flowers. Differentiation in various aspects of infection from the wild type plants would indicate that the effector played a critical role in the infection process and would warrant further investigation.

Conclusions

Prior to this research, no other *Microbotryum* generalist species had been transformed; in these studies, these species have been transformed both with pMvHyg, as had been done previously only for *M. lychnidis-dioicae* (Toh et al., 2016), and also with *cas9* under the control of a *Microbotryum* promoter. This opens up the *Microbotryum dianthorum* species complex to the research that has been conducted on *Microbotryum* species infecting *Silene*. Such future prospects may include transforming and disrupting genes using the CRISPR/Cas9 system of gene editing and then observing the infection progression to determine any phenotypic changes. Genes that would be disrupted or transformed would be selected through supporting bioinformatic analysis on *Microbotryum dianthorum* species that are currently underway in the Perlin lab and in the lab of a collaborator (Dr. T. Giraud, Université du Paris-Sud).

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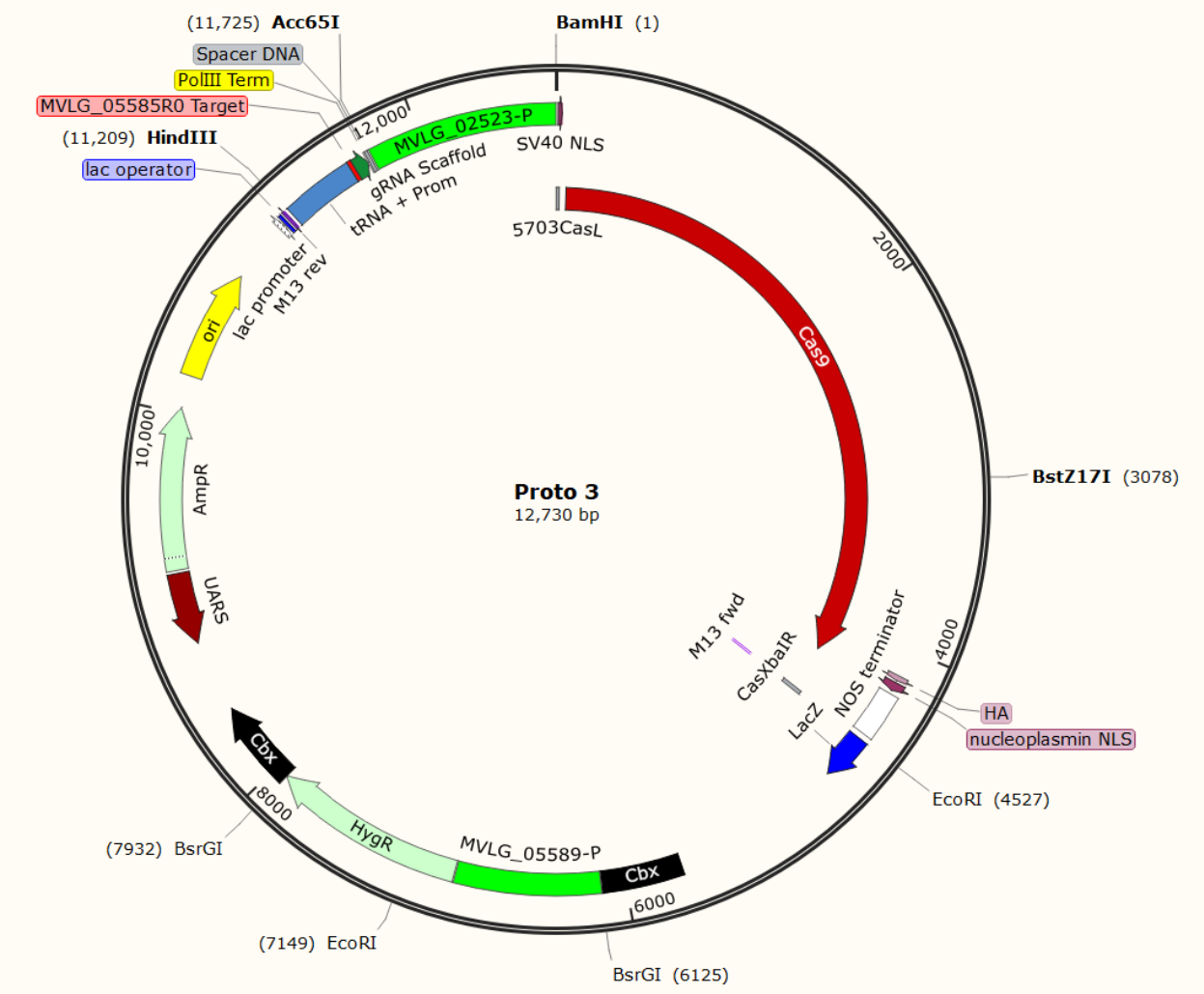
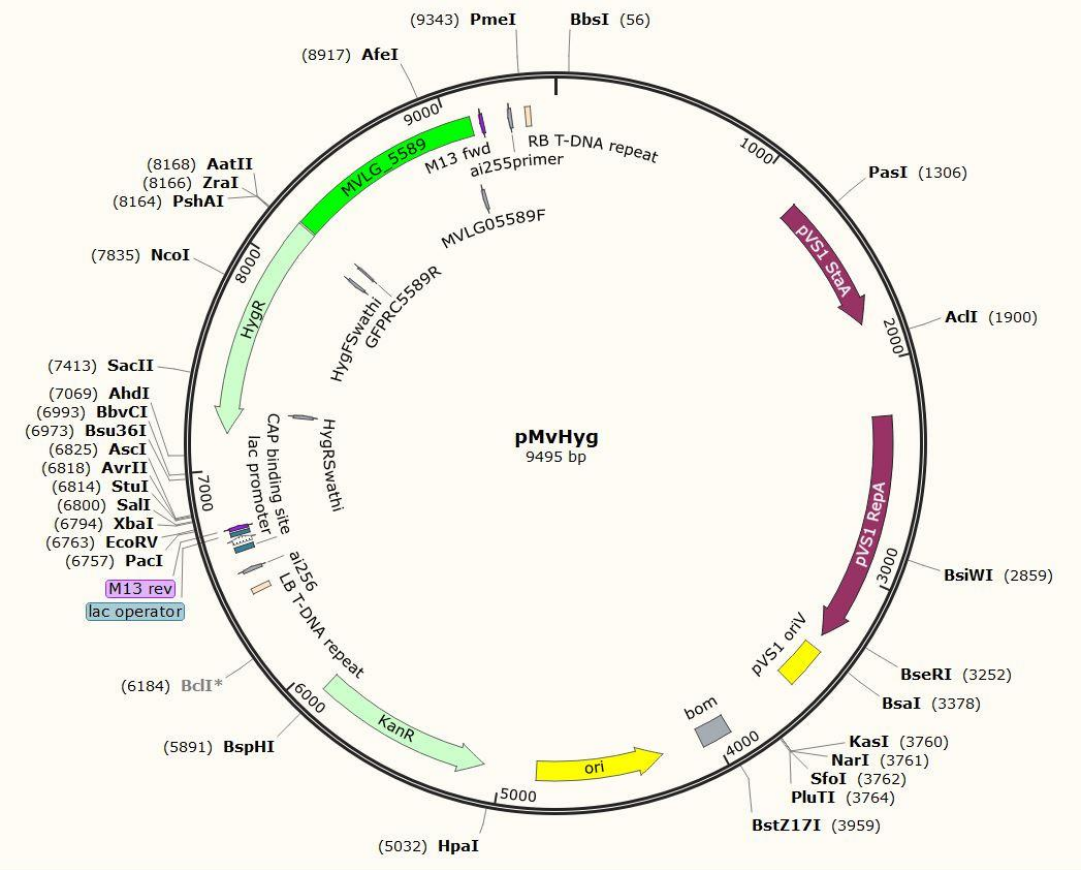
Appendix I: Additional Plasmid Maps



Plasmid map of Proto 3 plasmid. Cas9, gene that encodes the Cas9 protein; amplified during PCR using the primers 5703CasL and CasXbaIR as denoted on the map and yielded a 4.5 kbp band as expected (Fig. 4) (Toh et al., 2016).



Plasmid map of pMvHyg plasmid. HygR, gene conferring hygromycin resistance to *Microbotryum*, amplified during PCR using the primers HygFSwathi and HygRSwathi as denoted on the map and yielding an 877 bp band as expected (Fig. 5); KanR, gene conferring resistance to kanamycin in bacteria.

Appendix II: Additional Tables

Table of qRT-PCR of *cas9* expression. *MVLG_00180* expression was used to normalize expression data. Data correlate with Figure 7.

Sample	Media	Gene	Ct mean	ΔCt value	$2^{(-\Delta Ct)}$
P1A1 Wild type	PDA	*MVLG_00180*	21.7	N/A	N/A
		cas9	undetected		
	YPD	*MVLG_00180*	21.8	10.54	0.0007
		cas9	32.339		
P1A1 x pMvHyg5703Cas9	PDA	*MVLG_00180*	21.0	-1.62	3.07
		cas9	19.4		
	YPD	*MVLG_00180*	22.1	-1.90	3.74
		cas9	20.2		

Table of qRT-PCR of *cas9* expression. *MVLG_00180* expression data from the previous table was averaged and used to normalize expression data in lieu of *MVLG_00180* expression data for this test as denoted by an asterisk. Data correlate with Figure 8.

Sample	Media	Gene	Ct mean	ΔCt value	$2^{(-\Delta Ct)}$
MvDp 6D Wild type	PDA	*MVLG_00180*	21.5*	10.6	0.0007
		cas9	32.1		
	YPD	*MVLG_00180*	21.5*	10.5	0.0007
		cas9	32.0		
MvDp 6D x pMvHyg5703Cas9	PDA	*MVLG_00180*	21.5*	7.9	0.004
		cas9	29.4		
	YPD	*MVLG_00180*	21.5*	10.3	0.0008
		cas9	31.84		
P1A1 x 5589Cas9	PDA	*MVLG_00180*	21.5*	-2.2	4.6
		cas9	19.3		
	YPD	*MVLG_00180*	21.5*	-1.3	2.4
		cas9	20.22		
P1A1 Wild type	PDA	*MVLG_00180*	21.5*	N/A	N/A
		cas9	undetected		
	YPD	*MVLG_00180*	21.5*	9.6	0.0013
		cas9	31.1		
P1A1 x pMvHyg5703Cas9	PDA	*MVLG_00180*	21.5*	-1.8	3.39
		cas9	19.74		
	YPD	*MVLG_00180*	21.5*	-1.8	3.5
		cas9	19.7		

Table of qRT-PCR of *MVLG_05703* expression. *MVLG_00180* expression data from the first appendix table was averaged and used to normalize expression data in lieu of *MVLG_00180* expression data for this test as denoted by an asterisk. Data correlate with Figure 9.

Sample	**Media**	**Gene**	**Ct mean**	**ΔCt value**	**$2^{(-\Delta Ct)}$**
MvDp 6D Wild type	PDA	*MVLG_00180*	21.5*	6.9	0.009
		MVLG_05703	28.4		
	YPD	*MVLG_00180*	21.5*	7.5	0.006
		MVLG_05703	29.0		
MvDp 6D x pMvHyg5703Cas9	PDA	*MVLG_00180*	21.5*	7.6	0.005
		MVLG_05703	29.1		
	YPD	*MVLG_00180*	21.5*	8.5	0.003
		MVLG_05703	30.0		
P1A1 x 5589Cas9	PDA	*MVLG_00180*	21.5*	-8.3	318.7
		MVLG_05703	13.2		
	YPD	*MVLG_00180*	21.5*	-2.8	6.9
		MVLG_05703	18.7		
P1A1 Wild type	PDA	*MVLG_00180*	21.5*	-8.3	308.7
		MVLG_05703	13.2		
	YPD	*MVLG_00180*	21.5*	-5.2	36.5
		MVLG_05703	16.3		
P1A1 x pMvHyg5703Cas9	PDA	*MVLG_00180*	21.5*	-8.3	318.7
		MVLG_05703	13.2		
	YPD	*MVLG_00180*	21.5*	N/A	N/A
		MVLG_05703	undetected		