July 8, 2023

To: TACF External Grants Committee

From: Bruce Levine, University of Maryland

Subject: TACF External Grant 2019-3: Progress report

Summary: TACF External Grant 2019-3, provided funding to develop a high-efficiency CRISPR-mediated targeted mutagenesis system in *Cryphonectria parasitica* (Cp). Work under the grant demonstrated that the CRISPR/Cas9 system can be used to edit the Cp genome, and doing so is more efficient than using the prevailing homologous gene replacement (HGR) method. Of the two approaches used to apply CRISPR/Cas9 to Cp, the method that happened to work was the direct integration of the Cas9 endonuclease gene into Cp. I created a new strain, called DC9, in which the Cas9 gene was inserted at a harmless locus and is apparently expressed. When guide RNA matching sites in a target gene are combined with a donor DNA sequence to replace the target gene, it results in a higher (though still low) transformation percentage than when using donor DNA alone (the HGR method). Moreover, many of the guide RNA-induced transformed colonies contain no trace of the wild-type target gene. This is notable because using HGR alone almost always results in colonies containing both wild-type and transformed nuclei, which subsequently have to be isolated via single spore cultures, which adds several weeks to the process and can be troublesome. An attempt to use a different method to achieve CRISPR/Cas9 gene editing, involving a transiently expressed plasmid combined with synthesized guide RNA, did not succeed. The failure of this attempt does not mean that transient expression of Cas9 and guide RNA will not work with some modifications to the approach, and it merits further investigation Transient CRISPR/Cas9 gene editing methods offer the promise of much higher transformation efficiencies, the ability to make multiple edits in one transformation, and the creation of mutant Cp strains that carry no antibiotic resistance marker or any other lasting footprint, except the desired edits themselves. Establishing that CRISPR/Cas9 works in Cp is an important step, and suggests that other, more efficient methods will be feasible.

Project activities and results:

This is a report on the activities carried out, and results obtained under TACF External Grant 2019-3, *Developing a high-efficiency CRISPR-mediated targeted mutagenesis system in Cryphonectria parasitica,* awarded in October 2019, and continuing under no-cost extension was awarded until November 30, 2023. Progress on the project stopped for the better part of 2020 when Covid-19 restrictions kept me from the lab at the University of Maryland.

Program goals:

Under this grant, I proposed to develop a CRISPR/CAS9-based method for targeted mutagenesis in the chestnut blight fungus, C*ryphonectria parasitica (*Cp*).* The project had two goals:

1. Demonstrate CRISPR/Cas9-mediated targeted mutagenesis in Cp using transient expression of plasmid DNA carrying the Cas9 gene and target-specific guide RNA (either expressed in a plasmid or by means of *in vitro* synthesized guide RNA.)
2. To develop a "CRISPR-ready" *Cp* strain, with the Cas9 gene and promoter permanently integrated into the genome so that subsequent transformations will be even simpler, requiring only delivery of only guide RNA and any desired selection marker, reporter gene or novel inserted DNA sequence.

Goal 1: Cas9-mediated genetic modification by transient expression of plasmid DNA.

I made one unsuccessful attempt in January 2022 to genetically modify Cp (strain EP155) using a transiently expressed CRISPR/Cas9 system. The attempt involved co-transformation of Cp spheroplasts with the plasmid DNA and *in vitro* synthesized gRNA. The the plasmid, pFC332, obtained from Addgene (1), which expresses a fungal-optimized version of the Cas9 gene from *Streptococcus pyogenes*, and a hygromycin phosphotransferase gene (hph) that enables selection of transformed fungal colonies through the use of the antibiotic hygromycin. The guide RNA used were synthesized using the Invitrogen GeneArt Precision gRNA Synthesis Kit, designed to target two regions of the first exon of the CpSec66 gene, a non-essential gene which prior work (2) has shown to be relatively easy to knock out, and which results in a visually observable mutant phenotype.

Though the attempt was not successful, the approach is based on successful CRISPR/Cas9 transformations of other fungal species (1), and will likely succeed in future attempts with adjustments to the transformation conditions, or the amount of plasmid DNA and guide RNA used. To date, I have not had time to repeat the effort, because I have been working on Goal 2.

Goal 2: Cas9-mediated genetic modification by permanent integration of the Cas9 gene into Cp.

I succeeded with goal 2, creating "DC9," a strain of Cp derived from the DK80 strain (itself a knock-out strain derived from EP155). DC9 differs from its parent DK80 in that the Cas9 endonuclease gene is inserted in place of the non-essential Vic4-1 gene. (The Cas9 gene used was taken from the pFC332 plasmid, demonstrated in previous work to be functional in *Aspergillis* fungi.(1) The insert containing Cas9 also contains a

neomycin resistance gene to allow for the use of antibiotics to select positive transformants. I used Gibson assembly to insert the Cas9/Neomycin resistance insert with flanking sequences targeting it to the Vic 4.1 locus, which I chose because previous work showed that Vic 4.1 could be knocked out without impairing the fungus (3).

The resulting insert was 8.3 kb in length and too long to easily amplify by PCR. Instead I used the Gibson assembly process to fuse it to an off-the shelf plasmid, pUC19, for replication in bacteria. I included HindIII restriction enzyme cutting site sequences between the insert and the pUC19 backbone. Because the HindIII sequence is not found elsewhere in the insert or the backbone, this allowed me to use HindIII digestion of the resulting plasmid DNA to cut the insert out and separate it from the backbone by gel electrophoresis. This produced the large amounts (5-10 ug) of DNA fragments necessary to transform DK80 by HGR. The transformation was challenging, in part because the 8.3 kb insert was much longer than the 2.7 kb target locus. Numerous colonies survived that were resistant to the antibiotic G418 (a Neomycin class antibiotic), but several of these transformed colonies incorporated only the Neomycin resistance end of the insert but lost the middle of the Cas9 gene at the other end. However, I obtained two colonies, DC9-C and DC9-E, that contained the entire Cas9/Neomycin resistance insert, as confirmed by Sanger sequencing. I allowed these colonies to grow out and sporulate, and plated diluted spores on antibiotic medium to select for antibiotic-resistant colonies that only carried the Cas9/Neomycin resistance insert, but not the wild-type Vic 4.1 gene. I got two: DC9-E6 and DC9-E8. PCR tests and sanger sequencing of the Vic4.1 loci in these two cultures confirmed the Cas9 genetic sequence and Neomycin resistance sequence are present at the Vic4 locus, and that the wild-type Vic4 gene was absent. I single-spore subcultured DC9-E6 and -E8 through three generations and reconfirmed by PCR and Sanger sequencing that the Cas9/Neomycin resistance insert remained stably integrated. The culture I call “DC9” and used for subsequent CRISPR-mediated transformations is DC9-E6. I did not test directly for expression of the Cas9 protein in DC9, but inferred that it is expressed based on results described below.

My first attempt to show CRISPR in action using DC9 was in April 2022, and was not successful. I used guide RNA targeting two sites in the first exon of the Avr1 gene in Cp in an attempt to produce recognizably mutant colonies. Previous work (2) showed that deletion of this gene should result in mycelia with a notably abnormal phenotype. This, I hoped, would obviate the need to add any kind of reporter or marker gene to identify transformants – the changed phenotype itself would be the marker. I did indeed see two distinct colony phenotypes, but the two phenotypes were observed in the non-transformed controls as well, and PCR and Sanger sequencing of various colonies produced the identical Avr1 locus sequence regardless of phenotype. I concluded that the dual phenotype must have been due to an unrelated, spontaneous mutation in the DC9 culture from which I made the spheroplasts..

My second attempt to test DC9 targeted the CpSec66 gene. I had previously deleted this gene without CRISPR from the DK80 strain of Cp, using HGR (2). I performed three types of transformations in the DC9 background to knock out the CpSec66 gene:

1. One transformation used the standard HGR method in spheroplasts made from DC9. The gene disruption cassette used to knock out CpSec66, identical to the one I previously used in DK80, contained the hygromycin resistance gene (hph) flanked by ~500 base pair sequences that matched the flanking sequences of the CpSec66 gene.
2. A second transformation combined the hph gene disruption cassette with synthesized guide RNA targeting portions of the first exon of the CpSec66 gene. This would hopefully trigger the CRISPR/Cas9 system to make lesions at the guide RNA target sites and improve transformation efficiency compared to what is achievable with HGR alone.
3. The third transformation used DC9 spheroplasts using guide RNA only. Since no gene disruption cassette (donor DNA) was involved in this transformation, transformed colonies would not be resistant to hygromycin. Alternative means of identifying transformed colonies were necessary (discussed below). If successful, this method offered the possibility of transformation without leaving any antibiotic resistance or other footprint in the resulting mutants.

For the first two transformation methods, I did two comparisons:one in June 2022 and another in January 2023. Results in both cases indicated improved transformation efficiency through the use of guide RNA in the DC9 strain compared to when using donor DNA alone, but the improvement did not present itself in the manner expected. I had expected that CRISPR/Cas9-induced double stranded breaks in the genomic DNA at the target sites would result in a much higher frequency of transformation in the guide RNA-assisted transformation. However, the first attempt produced the exact same number of colonies whether or not guide RNA was added, and the second attempt produced slightly more than twice as many colonies when gRNA was used, which still represented a very low frequency for transformation (less than 1%) when compared to the total number of spheroplasts used. Improved efficiency presented itself in a different way – on both occasions about half of the transformed colonies produced with the addition of gRNA showed an immediate mutant phenotype. This is notable because HGR alone almost always (in my experience, always) produces colonies in which some nuclei are transformed and some are not (known as heterokaryon colonies). In CpSec66 knockouts, heterokaryon colonies show the wild-type phenotype, and can only be detected by PCR amplification of the target region, which will show two bands, one mutant length and one wild-type length. Heterokaryon colonies have to be induced to produce spores so that single-spore cultures can be isolated that only carry mutant nuclei (known as monokaryons). PCR tests confirmed that the antibiotic-resistant colonies produced with HGR only were all heterokaryons, as expected. For antibiotic-resistant colonies produced with the addition of gRNA, however, about half contained no trace of the wild-type CpSec66 gene. These monokaryons all showed the mutant phenotype. This eliminated the need to isolate single-spore cultures, and more

importantly, showed that something very different was happening when gRNA was introduced.

I compared two of the CRISPR-induced monokaryon knockouts to a CpSec66 knockout I had previously created using HGR alone (called dTG4A8). The three strains were all similar to each other and notably different from the DK80 parent strain in appearance, response to light, and virulence in chestnut seedlings (via small stem assay).

The January 2023 round of transformations included the third transformation method, in which gRNA but no donor DNA was introduced to DC9 spheroplasts. This produced tens of thousands of colonies that, since they had no antibiotic resistance selection marker, had to be screened by other means for the desired mutation. I did this in two ways:

1. Visual observation for mutant phenotype:. After transformation and regeneration of the spheroplasts into fungal colonies, I transferred 500 individual colonies randomly selected from regeneration medium plates to standard potato dextrose agar (PDA) plates, 25 colonies per plate. This left enough space between them that they could grow large enough to observe whether they had the wild-type or mutant phenotype before they grew into each other. Of the 500, none showed the mutant phenotype.
2. Amplicon sequencing: I added a layer of liquid growth medium to three regeneration plates, allowed the mycelia to grow for 48 hours, and collected pooled samples of mycelia from hundreds-to-thousands of colonies. I extracted genomic DNA from those pooled samples, then PCR amplified a 450 base pair segment of the genome containing both gRNA target sites from this pooled DNA and sent it to Azenta/Genewiz for Amplicon sequencing. Amplicon sequencing generates separate reads for tens of thousands of DNA fragments, which can be analyzed to determine the frequency of variants in the population of reads as well as the location of any frequently occurring mutations. Azenta's analysis of 83,965 reads in the gRNA-transformed sample showed mutations in only 0.67% of reads, compared to only 0.43% of 176,866 reads from an untransformed sample DC9 genomic DNA, which provides a rough baseline for the frequency of PCR errors or read errors. In the gRNA-transformed sample, some of the observed mutations were consistent with frame shift errors that could have been due to CRISPR-induced erroneous repairs near one of the gRNA target cut sites, but the incidence of these errors was extremely low. When I also uploaded the data files for analysis on the third party site CRISPR RGEN, it showed an estimated 0.0% indel rate within 20 base pairs of either target site.

During the course of the project, I made three attempts to produce an "EC9" strain, analogous to DC9, but in the EP155 background instead of DK80, which has an impaired non-homologous end-joining (NHEJ) function. If successful this could allow for CRISPR/Cas9 transformation of a wild-type strain without the benomyl-resistance footprint left over from when EP155 was transformed into DK80. DK80's impaired NHEJ function boosts the efficiency of HGR. However, the naturally error-prone NHEJ

function may be desirable in a CRISPR/Cas9 mediated system as it can be used to induce indels and frameshift errors at the guide RNA target site without the use of donor DNA. None of the efforts to make an EC9 strain have yet succeeded, but if it is possible in DK80, it is possible with EP155, given enough attempts.

My final activity under Goal 2 was to attempt another gene knockout in the DC9 background using both HGR alone and HGR combined with gRNA. This time I targeted the "TG6" gene which I had not been able to knock out in five previous attempts using HGR alone. (Like CpSec66, TG6 has a homologue which is upregulated in the invasive tissues of powdery mildew fungus. It has a signal peptide sequence and no predicted transmembrane domains suggesting that it may be secreted (2). Its small size and lack of close homology to other known proteins is typical of effector proteins pathogens use to disrupt the immune functions of their hosts (5).

The TG6 knockout succeeded in the transformation using both donor DNA and guide RNA, producing a single monokaryon colony, DC9dTG6-C1. PCR amplification and Sanger sequencing of the TG6 region showed that "C1" had incorporated the donor DNA (the hph marker gene), and no longer carried the wild type sequence, at the TG6 locus. The transformation that used donor DNA only produced 26 hygromycin-resistant colonies, at least some of which were shown by PCR testing to be heterokaryon colonies. This may mean that TG6 was not as difficult to knock out as I thought, but it remains a mystery why there were so many hygromycin resistant colonies in the DNA-only transformation, but only one in the gRNA-assisted transformation (all other transformation conditions were equivalent). Why did the addition of guide RNA suppress the heterokaryon colonies, and/or ectopic insertion events and/or other escapes seen in this and previous attempts to knock out TG6 using HGR alone? One possibility is that the large amount of guide RNA used in the transformation (20 ug each for two guide RNA fragments) may have somehow proven toxic to the spheroplasts. The method of using donor DNA combined with guide RNA may prove much more efficient if one uses far less gRNA than I did. This merits further exploration.

Conclusions and Next Steps:

This project demonstrated that CRISPR/Cas9 works in Cp and improves the efficiency of transformation, allowing the generation of monokaryon mutants in a single transformation step, without the need to isolate the mutant genome by single-spore culture as is normally required with HGR-based transformation. The significance of this is more than the possibility of saving a step in the transformation process. It is proof of concept that CRISPR/Cas9 gene editing works in Cp, and may offer a way to edit regions of the genome that have proven difficult to transform by standard HGR alone.

Direct integration of the Cas9 gene into Cp may not be the most efficient or desirable way to take advantage of the power of CRISPR/Cas9 in transforming Cp. Experiments in other fungal species using transient expression of Cas9 and/or gRNAs, or using custom synthesized ribonucleoproteins (RNPs) showed much higher transformation

efficiency than seen in this experiment (6,7,8). I used the direct integration approach because it was the one that happened to work first. Though this project provides proof of concept, further work using other approaches will be necessary to demonstrate whether the greatest potential benefits of using CRISPR/Cas9 to transform Cp are achievable.

Following are questions that remain to be answered and proposed approaches to answering them:

Can CRISPR/Cas9 be used transiently to induce mutations in Cp?

- Re-attempt transformation using the pFC332 or other Cas9 plasmids, either in combination with synthesized gRNA or using plasmids that also express the guide RNA sequence.
- Attempt transformation using *in vitro*-synthesized ribonucleoproteins (RNPs) available from such companies as IDT. This approach, demonstrated successfully in several other species of ascomycete fungi, takes more money (about $1,000-2000 per attempt) but saves time, eliminating the need for molecular cloning to construct plasmids.

Can transient CRISPR/Cas9-based gene editing eliminate unwanted footprints of antibiotic resistance in the mutant strains produced?

- Re-attempt transformation using pFC332 or other Cas9-bearing plasmids that also transiently express antibiotic resistance enzymes only as long as they persist in the resulting mutant colonies.

Can guide RNA alone induce mutations in CRISPR/Cas9-enabled strains of Cp?

- Attempt guide RNA-only transformation in a CRISPR/Cas9-enabled derivative strain of EP155, rather than of DK80. This may work better because CRISPR-induced mutations in the absence of any donor DNA depend on the error-prone NHEJ process, which is partially impaired in DK80 and DC9.

Can CRISPR/Cas9 be used to delete or modify genes that do not seem amenable to modification by HGR alone?

- Target intractable genomic regions using HGR + gRNA in DC9 spheroplasts, e.g. the complete AVR1 region.

Can the efficiency of CRISPR-mediated transformation be improved even further?

- The use of synthesized RNPs and chemically modified enzymes, oligos and reagents available from companies like IDT have been shown to greatly increase the efficiency of CRISPR-based transformations in other cell types. Though somewhat costly, they can greatly increase editing efficiency.

- CRISPR systems using the Cas12a endonuclease appear to cause more mutations in other eukaryotic organisms than Cas9 does.
- Some genes, such as the yeast Rad52 gene appear to increase the efficiency of homology directed repair in animal cells, and may do so in fungi. Fusion of this gene to the N terminus of the Cas9 protein has been shown to boost transformation efficiency several fold (9).

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