

Title: Developing a high-efficiency CRISPR-mediated targeted mutagenesis system in *Cryphonectria parasitica*

Summary:

CRISPR/Cas9 is a new technology for making precise edits in the genomes of subject organisms. CRISPR/Cas9 has been demonstrated in several species of filamentous fungi, but not in *Cryphonectria parasitica* (*Cp*). The benefits of CRISPR/Cas9 over other methods include its relative simplicity, high efficiency, and its ability to target multiple genes in one transformation. We propose to develop two types of CRISPR/Cas9 gene editing methods optimized for *Cp*: one through the insertion and transient expression of CRISPR/Cas9 constructs, and another involving the permanent insertion of the Cas9 endonuclease gene into the *Cp* genome to create a CRISPR-ready strain for high-throughput genetic studies.

Principal Investigator/Institutional Affiliation: Bruce Levine, PhD student, University of Maryland, Institute for Bioscience and Biotechnology Research.

Duration of project: 12 months

Total amount requested: \$4,600

Plasmids and sequencing	\$1000	\$1000
Reagents	\$2000	\$2000
Miscellaneous lab materials	\$1000	\$1000
One greenhouse bench for one year @ \$25/month	For small stem assay virulence testing of WT and CRISPR/CAS9-modified strains	\$600
TOTAL		\$4600

The PI will seek matching funding for this project from the John Z Duling Grant offered by the TREE Fund in 2019. The amount request from TACF is sufficient to support the objectives described in this application. Any additional funds received will be used to move from the demonstration/proof of concept phase to knocking out both known and suspected pathogenicity genes in *Cp*.

How and when funds will be spent:

This application requests funding for materials and greenhouse space only, and the budget provided above represents the applicant's best estimate of material costs. No funds for labor, tuition or student stipends are requested. The applicant will request reimbursement approximately monthly for project-related charges for materials, greenhouse space or sequencing services, with total amounts not to exceed the budget provided above. We anticipate requesting a large portion of the funds early in the project, during the materials acquisition phase. Sequencing and greenhouse space costs will be continuing small expenses.

Narrative:

The American Chestnut Foundation (TACF) uses Chinese chestnut (*Castanea mollissima*) as the source of resistance in its program to breed blight-resistant trees for reintroduction to eastern U.S. forests. This resistance has long been known to involve multiple genes. The most recent genetic evidence (Westbrook et al, 2019) found at least nine genomic loci that correlated with resistance in a Chinese-American hybrid population. This finding suggests that TACF's backcross breeding program, designed for a three-gene model, is unlikely to produce the desired reliably highly-resistant, mostly-American hybrids for reintroduction into American forests. Better methods, such as marker-assisted selection, and/or the introduction of novel forms of resistance, will be required to achieve TACF's goals.

Whether one screens for naturally-occurring resistance genes or seeks to introduce novel ones, it is important to understand the molecular basis of the interaction between the host (*Castanea* species) and the pathogen (*Cp*). For the chestnut blight pathosystem, the host is difficult to transform and clonally propagate, even compared to other woody plants. The pathogen, however, is highly amenable to genetic manipulation, including targeted mutagenesis, and thus is a good subject for reverse genetic studies (the deletion or impairment of specific genes of interest) that can shed light on chestnut blight and other diseases caused by related plant pathogenic fungi. Reverse genetics in *Cp* is facilitated by the availability of an annotated reference genome for the EP155 strain of *Cp*, a well-established method for homologous gene replacement (HGR) in *Cp* (Churchill et al 1990), and a mutant strain, DK80, which has an impaired non-homologous end-joining DNA repair pathway, greatly improving the efficiency of genetic transformation. HGR has been used to study the role of numerous *Cp* genes (Chen et al, 2011; Choi et al, 2005; Chung et al 2006; Jacob-Wilk et al, 2009; Kazmierczak et al, 2005; Kim et al, 2004, Moretti et al, 2014), as well as to produce "superdonor" *Cp* strains that are capable of overcoming vegetative incompatibility-type barriers and transmitting chestnut hypovirus to any *Cp* mycelium (Zhang and Nuss, 2016).

Despite its usefulness, HGR has limits. It is time-consuming, difficult to use when deleting multiple genes (Zhang et al, 2016), and sometimes results in off-target insertion of selective markers, producing false positive results. It is not suitable for disruption of multiple copy genes, and may not work at all for some genes for reasons that are not clear. For example, the applicant attempted to knock out six suspected pathogenicity-related genes in *Cp* using HGR (Levine, 2019), but was only successful with one, the *Cp* homologue of the *Saccharomyces cerevisiae* translocase gene, *Sec66* (*CpSec66*). Transformation efficiency with HGR is also low unless one uses the DK80 (or any other similarly impaired) strain, but DK80 itself exhibits rapid senescence and must be frequently refreshed from single spore cultures. Thus, even though HGR works for transforming *Cp*, there is room for improvement, and the emergence of the CRISPR/Cas9 system as a new gene editing tool offers great promise.

The CRISPR/Cas9 technology has been successfully used to make targeted lesions, deletions, and insertions into the genomes of several species of filamentous fungi, including ascomycete fungi closely related to *Cp* (Fuller et al, 2015, Li et al 2018, Wang et al, 2018), but not yet in *Cp* itself. The CRISPR/Cas9 system depends on targeting of the Cas9 endonuclease to specific DNA sequences by means of small guide (sg) RNA. The Cas9 endonuclease makes a double-stranded break (DSB) precisely at the targeted sequence, which is then repaired by fungal homologous or non-homologous DNA repair systems. If donor DNA, with homology to the flanking sequences of the DSB site is introduced with the Cas9 complex, it can result in insertion of the donor sequence. If homologous DNA is not present, the non-homologous system will repair the break, causing disruption to the target sequence, including possible frameshift mutations that result in premature stop codons/non-functional genes. Mutagenesis

by CRISPR/Cas9 can also be done in a manner that leaves no selective marker or other footprint in the subject genome.

The methods used for CRISPR/Cas9-mediated transformation in other fungi have varied widely, depending on the idiosyncrasies of the fungus being transformed. For *Cp*, the applicant intends to first use PEG-mediated transfection of *Cp* spheroplasts, as this method works well for HGR. If necessary, the applicant will move on to other methods used for other fungi, such as electroporation of spores, or Agrobacterium-mediated transformation of spores or spheroplasts. The applicant will initially use the fungal promoter *gpdA* or *tef1* to express Cas9, and if unsuccessful in achieving stable expression of Cas9, will use other promoters that have been successful in transformations of species closely related to *Cp*.

Short and Long-term Goals of this Project:

The long-term goal of this project is to understand key pathogenicity mechanisms of *Cp* for developing more efficient resistance in American chestnut. This proposal seeks to develop a *Cp*-optimized CRISPR/Cas9-based method for targeted mutagenesis. Establishment of this gene-editing technology for *Cp* will not only facilitate genetic studies on chestnut blight, but may also make *Cp* a model fungus for studying plant pathogenic ascomycete fungi, which would have benefit the chestnut research community and beyond.

In order to establish a *Cp*-optimized CRISPR/Cas9 system for efficient targeted mutagenesis, within the project period, the applicant has two specific objectives:

1. Develop and demonstrate an efficient method to deliver CRISPR/Cas9 complexes into *Cp* for targeted mutagenesis
2. Optimize delivery of small guide RNAs to a “CRISPR-ready” *Cp*

To accomplish objective 1, the applicant will first use PEG-mediated transformation to introduce plasmid DNA and linear DNA, each containing the necessary combination of the CAS9 gene, its promoter and nuclear localization signal, selective marker(s) and guide RNA-encoding sequences, to separate batches of *Cp* spheroplasts. We will compare the ease and efficiency of targeted mutagenesis for the two types of “tool” delivery.

We will attempt both gene disruption and gene insertion using CRISPR/Cas9. For targeted disruption, we will choose *Cp* genes whose disruption is known (from previous knockout studies using HGR) to produce visually obvious growth defects. Candidate genes include *Cp*pk1 (Kim et al 2005), *Cp*pk2 (Moretti et al 2014) or *Cp*lc1 (Chung et al 2006).

For CRISPR/cas9-mediated gene insertion, we plan to reintroduce *Cp*Sec66, translationally in-frame fused with green fluorescent protein (GFP), to the dTG4A-8 strain in which *Cp*Sec66 was deleted by the applicant by using HGR (thesis), and which the applicant has in his possession. Because dTG4A-8 strain has a noticeable phenotypic abnormalities (sparse disorderly growth, loss of pigment, loss of zonal growth pattern, and reduction in virulence) restoration of normal growth in dTG4A-8+CpSec66+GFP will validate the biological function of *Cp*Sec66 and report correct gene insertion. The GFP tag will also enable us to examine sub-cellular localization of the CpSec66 protein. Successful transformants will be selected on medium containing the appropriate antibiotics and regenerated through three generations of single spore cultures to confirm the stability of the CRISPR/CAS9-induced mutations. Stable

transformants will be analyzed by PCR and sequencing to examine (1) whether the targeted genes are mutated, (2) whether *CpSec66-GFP* is precisely inserted, and (3) whether the CRISPR/CAS9 transgene is stably integrated into the fungal genome or only transiently expressed.

To accomplish objective 2: the applicant will use HGR to precisely insert the *Cas9* gene expression cassette along with the hygromycin selection marker into an intergenic region of the *Cp* genome or replace an endogenous gene whose deletion has no obvious negative impact on fungal growth and development, such as vegetative incompatibility locus genes (Zhang et al, 2016). Once stably introduced into the *Cp* genome, and confirmed over three generations of single spore subcultures, the presence and expression of the *Cas9* transgene will be examined. If a *Cas9*-stabilized *Cp* strain is obtained, we will introduce sgRNAs (targeting the same reporter genes as for objective 1) by a variety of methods, including direct exposure of spores to synthetic sgRNAs, or PEG-mediated transformation of spores or spheroplasts with the corresponding DNA constructs. We will compare the ease and efficiency of targeted gene mutagenesis via different methods of sgRNA delivery. We will also test whether the *Cas9* gene itself has any effect on fungal fitness, phenotype or virulence, by growing a +*Cas9 Cp* strain, without sgRNA sequences, side-by-side with its wild-type parent strain, under a variety of *in vitro* and *in planta* conditions.

Timeline, showing start and completion dates for each goal:

October 2019	Research and material acquisition/preparation. Harvest of seeds for <i>in planta</i> assays, and placement in storage
November 2019	Transformation and regeneration of EP155 and DK80 spheroplasts with CRISPR/Cas9 constructs
December 2019- January 2020	Culture and subculture of putative, <i>Cas9</i> mutant strains
January 2020	Planting of seeds in greenhouse for <i>in planta</i> Assays. Continued culture/subculture of Mutants expressing <i>Cas9</i>
February 2020	PCR and sequencing-based confirmation of successful transformation. Design and construction of sgRNAs

March 2020	Use of CRISPR/Cas9 methodology to disrupt Cppk1, Cppk2 or Cplc1 genes in Ep155 spheroplasts, and to insert the CpSec66 + GFP sequence into the CpSec66 mutant strain dTG4A-8. Culture and sub-culture of CRISPR/Cas9-generated knockout-out and knock-in mutants.
April 2020	Continue to sub-culture CRISPR/Cas9-generated knockout-out and knock-in mutants. PCR and sequence based confirmation of successful transformation.
May 2020	Begin <i>In vitro</i> and comparison of EP155, EP55 + Cas9 and CRISPR/Cas9-generated EP155 mutants.
June 2020	Begin small stem assay <i>in planta</i> comparison of EP155, EP55 + Cas9 and CRISPR/Cas9-generated EP155, DK80 and dTG4A-8 mutants.
July – September 2020	Data collection and write-up

How results will be measured and reported:

CRISPR/Cas9 transformation will be confirmed by PCR and sequencing-based analysis. Successful gene knockout and knock-in will be observed visually (and microscopically for re-introduction of CpSec66 to dTG4A-8) and confirmed by PCR and/or sequence-based analysis.

Fungal phenotype will be observed visually. Fungal growth *in vitro* will be measured by comparison of biomass accumulated on cellophane over growth media. Fungal virulence will be measured *in planta* by small stem assay in American chestnut seedlings with data collected on canker length, qualitative canker ranking, and days of survival prior to stem wilt distal to the inoculation site.

Results from this proposed research will be published in a peer reviewed scientific journal, with protocols made publicly available to facilitate the adoption of *Cp* as a model fungus for reverse genetic studies. If attempts to create a CRISPR-ready *Cp*+CAS9 strain are successful, the applicant will make this material available to TACF-affiliated researchers upon request.

Curriculum Vitae: (See attached)

Conflict of Interest statement: The PI is member of, and donor to TACF, and a member of the Board of Directors of the Maryland Chapter of TACF. The PI sees no conflict of interest arising from the projects described above.

References:

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- Jacob-Wilk, Debora et al (2009) Silencing of Kex2 Significantly Diminishes the Virulence of *Cryphonectria Parasitica*. *Molecular Plant-Microbe Interactions* 22, no. 2: 211–21. <https://doi.org/10.1094/MPMI-22-2-0211>.
- Kazmierczak, Pam et al (2005) A Hydrophobin of the Chestnut Blight Fungus, *Cryphonectria Parasitica*, Is Required for Stromal Pustule Eruption." *Eukaryotic Cell* 4, no. 5: 931–36. <https://doi.org/10.1128/EC.4.5.931-936.2005>.
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- Levine, B (2019) Identifying Highly-conserved Pathogenicity Genes in Chestnut Blight and Powdery Mildew Fungi as Targets for Novel Forms of Resistance. Master thesis published on ProQuest (Embargoed until April 20, 2020. Copies available upon request).
- Li Jingtao et al (2018) Introduction of large sequence inserts by CRISPR-Cas9 to create pathogenicity mutants in the multinucleate filamentous pathogen *Sclerotinia sclerotiorum*. *MBio* 9:e00567-18. <https://doi.org/10.1128/mBio>.

Moretti et al. (2014) Functional Characterization of the Three Mitogen-Activated Protein Kinase Kinases (MAP2Ks) Present in the *Cryphonectria Parasitica* Genome Reveals the Necessity of Cpkk1 and Cpkk2, but Not Cpkk3, for Pathogenesis on Chestnut (*Castanea* Spp.) *Molecular Plant Pathology* 15, no. 5: 500–512. <https://doi.org/10.1111/mpp.12111>.

Nødvig CS et al (2015) A CRISPR-Cas9 System for Genetic Engineering of Filamentous Fungi. *PLoS ONE* 10(7): e0133085. doi:10.1371/journal.

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Westbrook, J.W., et al (2019). Genomic selection analyses reveal tradeoff between chestnut blight tolerance and genome inheritance from American chestnut (*Castanea dentata*) in (*C.dentata* x *C. mollissima*) x *C. dentata* backcross hybrids. BioRxiv. <https://www.biorxiv.org/content/10.1101/690693v1>

Yamato, Tohru et al (2019) Single crossover-mediated targeted nucleotide substitution and knock-in strategies with CRISPR/ Cas9 system in the rice blast fungus. *Nature.com Scientific Reports* 9:7427 | <https://doi.org/10.1038/s41598-019-43913-0>

Zhang, Dong-Xiu, and Donald L. Nuss (2016) Engineering Super Mycovirus Donor Strains of Chestnut Blight Fungus by Systematic Disruption of Multilocus Vic Genes. *Proceedings of the National Academy of Sciences* 113, no. 8: 2062–67. <https://doi.org/10.1073/pnas.1522219113>.

Zhang, Dong-Xiu et al (2013) Simple and Efficient Recycling of Fungal Selectable Marker Genes with the Cre-LoxP Recombination System via Anastomosis. *Fungal Genetics and Biology* 61: 1–8. <https://doi.org/10.1016/j.fgb.2013.08.013>.

Curriculum Vitae

Bruce Jonathan Levine

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Educational Background

- 2019-present PhD student in Plant Science, with research focus on plant interactions with fungal pathogens. Institute of Bioscience and Biotechnology Research, University of Maryland, College Park, MD
- 2016-2019: Masters degree in Plant Science with research focus on plant interactions with fungal pathogens, University of Maryland, College Park, MD
- 1995-1996 Graduate-level training program in Economics, U.S. Department of State, Foreign Service Institute, Arlington, VA
- 1982-1986 B.A. in East Asian Studies from Wesleyan University, Middletown, CT

Continuing Education

Correspondence Course in Forestry, University of Maryland Agricultural Extension, Completed in 2005

Teaching Experience

Teaching Assistant, Food Security and Crop Protection, UMD College Park, August-December 2018

Teaching Assistant, Woody Plants of the Mid-Atlantic, UMD College Park, August-December 2017

Teaching Assistant, Environmental Science and Policy Capstone course, UMD College Park, August -December, 2016.

Board Memberships

Member of the 5-person Tree Commission, City of Takoma Park Maryland. (The Commission is an appellate and advisory body established under the City Tree Ordinance.)

Member of the Board of Directors, Maryland Chapter of the American Chestnut Foundation (MDTACF), 2013-present. President of MDTACF 2013-2015, Vice President 2015-2016.

Member of the Board of Directors of the American Chestnut Foundation, 2013-2016

Publications

“Seed stratification is not required for germination but is important for seed survival, “ (Co-authored with Laura Barth), published in *Chestnut*, Issue 2, Vol. 33, Spring 2019

"Identifying Highly-conserved Pathogenicity Genes in Chestnut Blight and Powdery Mildew Fungi as Targets for Novel Forms of Resistance, “ Bruce J. Levine Masters Thesis, May 2019. Embargoed until April 20, 2020. Copies available upon request.

“There’s Something in the Bark: Constitutive Defenses in Chinese Chestnut,” published in *Chestnut*, Issue 1, Vol. 33, Winter 2019

“Small Stem Assays may be a Reliable Screening Tool for Testing American Chestnut Resistance to *Cryphonectria parasitica*,” (Co-authored with Tom Saielli), published in *Chestnut*, Issue 1, Vol. 33, Winter 2019

“A look at Chestnut Mold in Maryland,” published in *Chestnut*, Issue2, Vol. 32, Spring 2018

“Insights from Chinese research on chestnut blight,” published in *Chestnut*, Issue 2, Vol. 31, Spring 2017

“Late Emergence and Multi-stems,” published in *Chestnut*, Issue 1 volume 32, September 2016.

Authored articles for the American Chestnut Foundation’s newsletter on October 2005 and May 2006 on cultivated and wild chestnut in China, providing information on Chinese chestnut trees that is not normally observable in the United States.

Translations

Translated key Chinese research papers on chestnut blight from Chinese into English for the American Chestnut Foundation, including:

- “Evaluation of the Resistance of Chinese Chestnut Cultivars to Chestnut Blight,” by Qin Ling et al, *Journal of Fruit Science*, 2002
- “Induced changes in the activity of enzymes associated with *Cryphonectria parasitica*.” Liu Debing et al, *Advanced in Horticulture*, Vol. 5, 2002
- “A study of chestnut blight pathogenicity, and discussion of the pathogen as a hybrid system.” Zhang Rongfeng et al, *Journal of Zhenjiang Forest Science and Technology*, Vol. 18, No. 5, June 1998
- “Variation in pathogenicity of *Cryphonectria parasitica* strains from different regions.” Zhang Dan and Song Xiaobin, *Journal of Northwest Forestry University*, Vol. 31, No. 3, 2016
- “Biological Characteristics of *C. Parasitica* Isolates with Difference Levels of Virulence.” Zhou Erxun et al, *Acta Pythopathologica Sinica*, Vol. 26, No. 3, 1996

Employment History

Foreign Service Officer. U.S. Department of State, 1990-2016