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August 30, 2023

The American Chestnut Foundation

Proposal Title:	Improving the efficiency of CRISPR-based gene editing in Cryphonectria parasitica
UMD Proposal Number:	77367
UMD Principal Investigator:	Bruce J. Levine
UEI:	NPU8ULVAAS23
EIN:	52-6002033

To whom it may concer:

Please find enclosed the above referenced proposal submitted on behalf of the University of Maryland (UMD) and signed by an Authorized Representative. A UMD Proposal Number has been assigned, which you may use to reference this proposal in any future communication with our office. The budget request is in the amount of $10,000.

UMD acknowledges that Bruce J. Levine is identified by name as the Principal Investigator (PI) at UMD and the PI intends to carry out all responsibilities identified in the attached proposal. UMD believes the proposed project is fundamental research and does not require that we obtain an export license under Export Control Regulations. UMD recognizes that any award resulting from this proposal will include terms and conditions required by the sponsor that are appropriate for a State Institution of Higher Education; that are required by applicable law or regulation on the date of execution; and any other mutually acceptable terms and conditions, except those inconsistent with Uniform Guidance (2 CFR Part 200) or those not allowed under Maryland state law or contrary to the Maryland Attorney General's guidance. UMD reserves the right to negotiate the terms and conditions of any resulting award.

Please direct any technical questions regarding this proposal to Bruce J. Levine at levinebj@umd.edu. Administrative questions should be directed to Josh Kiner at jkiner@umd.edu.

UMD looks forward to collaborating with The American Chestnut Foundation on this project.

Sincerely,

Katie McKeon

Katie McKeon
Associate Director

Title: Improving the efficiency of CRISPR-based gene editing in *Cryphonectria parasitica.*

Summary:

Under a previous grant, I demonstrated that the CRISPR/Cas9 system can be used to increase the efficiency of genetic modification of the chestnut blight fungus (*Cryphonectria parasitica* – Cp) compared to previously prevailing methods (11). I now propose to use synthesized ribonucleoproteins (RNPs) to boost genetic modification efficiency even further with a view to developing a high-throughput system for conducting reverse genetic studies in Cp. Will attempt to use RNPs method to knock out four candidate effector proteins recently identified in Cp (14), first individually, and then in single transformations simultaneously targeting multiple genes. Such studies can help reveal fungal genes responsible for Cp's unique virulence against chestnut, which in turn can lead to the discovery of naturally-occurring or novel host genes for resistance to the disease.

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

Duration of project: 12 months

Total amount requested: $10,000

Synthesized ribonucleoproteins (RNPs) and associated materials. Miscellaneous lab materials, reagents and sequencing services	Customized RNP components and associated reagents from IDT corp. for 4 transformations, estimated at $2000 per transformation.	$8,800
Two greenhouse benches for one year @ $50/bench per month	For small stem assay virulence testing of WT and modified Cp strains	$1,200
TOTAL		**$10,000**

The PI does not expect and will not seek funding from UMD or any department thereof. Support for grant activities may come through a USDA/NIFA competitive grant for similar but more systematic work if an application, now being prepared by Dr. Shunyuan Xiao, the PI's academic advisor at UMD/IBBR, is approved.

Short and Long-term Goals of this Project:

The short term goal of this project is to test synthesized RNPs to make targeted edits in the Cp genome. Target genes will be selected in consultation with TACF science staff based on bioinformatic screening of the Ep155 genome for candidate effector proteins. We propose beginning with 4 effectors, to be knocked out individually. We will then test the possibility of multiplex gene editing by attempting multi-gene knockouts in a single RNP-based transformation step. Should RNPs fail, we will fall back to attempting these knockouts using the CRISPR-aided HGR process developed under my previous grant.

If RNP-mediated transformation succeeds, I will seek to optimize transformation efficiency, decrease the time required for transformation, and minimize costs per transformation to establish a high-throughput approach to future gene edits in Cp.

The longer term goal of this project is to explore a longer list of candidate effectors and/or pathogenicity-related genes in Cp that may help reveal important details about the Cp-chestnut pathosystem. The Xiao Lab at IBBR is currently applying for USDA/NIFA funding for broader and more systematic research that will overlap this proposal, and cover the cost of follow-on activities, such as identifying the substrates in chestnut upon which fungal effector proteins act.

All knockout strains developed as part of this project will be tested for effects on fungal virulence by small stem assay of Chinese and American chestnut controls in seedlings maintained at the UMD greenhouse in College Park.

Narrative:

The American Chestnut Foundation (TACF) uses Chinese chestnut (*Castanea mollissima)* as the source of resistance in its program to breed trees resistant to the chestnut blight fungus, *Cryphonectria parasitica* (Cp), for reintroduction to eastern U.S. forests. Advanced hybrids have shown more variation in blight resistance, and lower blight resistance overall than expected. QTL analysis of TACF's hybrid population has revealed multiple loci for resistance on every chromosome, none of which appear to be responsible for much variation in resistance by themselves (1). The implications of this are that conventional breeding that depends on random genetic recombination may not be a feasible means of producing a population of trees which are highly American in character but also inherit high levels of resistance from Chinese chestnut. 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.) Reverse genetics in Cp is facilitated by the availability of an annotated reference genome for the EP155 strain of Cp, and an established method for homologous gene replacement (HGR) (2). HGR has been used to study the role of numerous Cp genes (3-9).

Deleting genes from the pathogen's genome provides a way to test whether candidate genes play a role in virulence. If the deletion of a particular gene results in reduced virulence in the host, we can trace how it contributes to disease. Methods such as proximity labeling or yeast two-hybrid tests can tell us what a secreted pathogen protein interacts with in the host, and potentially reveal specific genes responsible for susceptibility or resistance in the host – information that would be invaluable when selecting parents for breeding. Fungal genes revealed to play a role in virulence can also be good targets for novel forms of host resistance using host induced gene silencing or other methods. The development of the promising Darling 58 transgenic chestnut, for example, began with the deletion of the Oah1 gene in Cp (which is involved in the synthesis of the fungal toxin oxalic acid), which resulted in

greatly reduced virulence in chestnut (10). Whether the goal is to discover naturally occurring host genes responsible for resistance or susceptibility to chestnut blight, or to develop novel forms of resistance, the discovery of fungal genes involved in pathogenicity is a vital first step.

HGR has drawbacks, however. It is relatively inefficient, can only accomplish one gene edit at a time, and sometimes simply fails. Under a previous TACF external grant, the applicant improved on the HGR method by using the power of CRISPR/Cas9-mediated gene editing. By integrating the Cas9 endonuclease gene into the Cp genome, the applicant demonstrated that CRISPR/Cas9 works in Cp, producing a larger number of transformed colonies, and dispensing with the time consuming step of separating transformed and non-transformed nuclei through single spore isolation (11).

CRISPR/Cas9 research in other fungal species suggests that there is further room for improvement. Specifically, transformation rates achieved on other fungal species were several orders of magnitude higher than what could be obtained with a traditional HGR approach. Some such results were achieved through the use of introduced plasmids that transiently express the components of the CRISPR/Cas9 system within fungal protoplasts, but the highest efficiencies were achieved by introducing custom synthesized ribonucleoproteins (RNPs) to the fungal protoplasts (12). RNPs edit the target genome, but do not replicate and leave no trace of their presence besides the edit they make.

Having already shown that CRISPR/Cas9 works and improves efficiency of genetic transformation in Cp, I am now proposing to try the RNP approach to see how high a level of transformation efficiency can be achieved, and whether more than one edit can be made in a single transformation. Other labs at UMDhave used custom RNPs produced by IDT to conduct similar experiments on other species of fungi, and have had excellent results (13).

Meanwhile, the application of genomics to the Cp-chestnut pathosystem has advanced to the point that we can begin to select candidate fungal genes that are likely to interact directly with chestnut defenses. Host-specific plant diseases like chestnut blight are often the result of effector proteins (proteins that interfere with host defenses) produced by the pathogen. Effectors are secreted, and typically small, rich in cysteine and have little sequence homology to other known proteins. Several software tools are now available to screen pathogen genomes for genes likely to encode effectors, and such analysis has revealed that the Cp genome is rich in effectors (14). An examination of candidate effectors in Cp compared to those of closely related fungi, have even produced a relatively short list candidate effectors found uniquely in Cp (14). Such effectors would be good targets for deletion as we attempt to dissect the molecular interaction between Cp and its hosts.

This proposal intends to kill two birds with one stone: to 1) test the use of synthesized RNPs to perform CRISPR/Cas9 gene editing in Cp, and 2) test whether the deletion of certain candidate effector protein-encoding genes changes Cp's virulence in chestnut. Initial targets for deletion will be putative effector-encoded genes identified by Stauber et al (14) as being unique to Cp.

Timeline

Nov. 2023	Choose candidate effector (CE) genes 1-4 in consultation with TACF science staff

Dec. 2023	Create gene disruption constructs for CE1-CE4 using overlapping PCR or Gibson Assembly
Jan. 2024	Plant 150 each of Chinese and American chestnut seeds at UMD greenhouse for use in small stem assays
Jan. 2024	Design 2 gRNAs each for CE1 - CE4. Order RNP materials from IDT.
Feb. 2024	Transformation of EP155 and DK80 spheroplasts with CE1 - CE1 RNPs (one target gene per transformation)
Feb. 2024	Amplicon sequencing of transformed cultures to estimate transformation efficiency
(Feb. 2024)	(If RNP transformation fails) Perform CE1-CE4 knockouts in DC9 background using CRISPR-aided HGR.
Mar. 2024	Isolation/PCR testing/and sanger sequencing confirmation of putative transformed strains
May 2024	Begin small stem assays (>= 15 seedlings per treatment) to compare knockout strains to WT in both Chinese and American chestnut
June 2024	Attempt multiplex RNP transformation of EP155 and/or DK80
July 2024	Collect first (45 dpi) SSA results
Sept. 2024	Collect second (90 dpi) SSA results

How results will be measured and reported:

- Success of transformations will be determined through the use of antibiotic selection markers (transformed colonies will be antibiotic resistant.) Transformation genotype will be confirmed by PCR testing and, if necessary, Sanger Sequencing.
- Transformation efficiency of RNP transformations will be measured by Amplicon Sequencing (Azenta Genewiz)
- Effect of gene deletion on virulence will be determined by small stem assay in Chinese and American chestnut seedlings, based on the length of cankers at fixed points in time (45 and 90 days post-inoculation) from the inoculation mid-point down, and by the 5 point rating scale previously used by the applicant for small stem assays.
- The applicant will freely provide results and data to TACF and its collaborators for inclusion in published articles in peer reviewed journals, and will prepare a plain English article for non-scientific audiences on project results for publication in Chestnut magazine.
- Applicant may publish detailed scientific results in an appropriate peer-reviewed journal.

Breakdown of how and when funds will be spent:

This application requests funding for materials, commercial services and greenhouse space only, and the budget provided above represents the applicant's best estimate of material costs. Greenhouse fees will occur monthly. All other fees will arise as needed during the course of the project, on a reimbursable basis. No funds for labor, tuition or student stipends are requested.

The table below lists estimated maximum costs for various materials and services that will be consumed in the course of the project, but the amount actually spent on each will depend on how project activities unfold. (Materials left over from the previous TACF grant can be used for the work proposed in this grant,)

RNP custom components and reagents from IDT	For four transformations	$2000 per transformation. Up to $8000 total.
Greenhouse fees	Two benches at $50 per bench per months	$1200
QuickDNA Fungal/Bacterial Microprep Kit	For DNA extraction	$196 for new kit. Less for replacement supplies.
PCR reagents	For PCR using LiTaq 2x mastermix or Q5 high-fidelity polymerase.	$183 for LiTaq. $122 for Q5.
Glucanex or Driselase enzyme	For spheroplast preparation	$200
GeneArt Precision gRNA synthesis kit	For making guide RNA for CRISPR-aided gene editing	$670 for new kit. Less for replacement supplies.
Primers	Each transformation requires approximately 10 small primers and 2-4 long primers.	$240 per transformation. Up to $960 total.
Sanger sequencing, whole plasmid sequencing	2-4 sequencing activities per transformation	Est $40 per transformation. Up to $160 total.
Amplicon sequencing	2 samples sequenced per transformation	$85 per sequence. Up to total of $680.

Curriculum Vitae: (See attached)

Conflict of Interest statement: The PI is a donor and member of the Board of Directors of TACF, a member of the TACF Science and Technology Committee, current chair of the TACF Chapters Committee, and Vice President of the Maryland Chapter of TACF. The PI sees no conflict of interest

arising from the projects described above, but is willing to step down from any TACF positions if TACF leadership so requests.

References:

(1) Westbrook, Jared, (2022) Science Update to the Board of the American Chestnut Foundation

(2) Churchill, A. C. L. et al (1990) Transformation of the fungal pathogen Cryphonectria parasitica with avariety of heterologous plasmids. *Current Genetics*, *17*(1), 25–31. https://doi.org/10.1007/BF00313245

(3-9) 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).

(3) Chen, Min-Mei et al, (2011). CYP1, a Hypovirus-Regulated Cyclophilin, Is Required for Virulence in the Chestnut Blight Fungus. *Molecular Plant Pathology* 12, no. 3: 239–46. https://doi.org/10.1111/j.1364-3703.2010.00665.x.

(4) Choi, Eun-Sil et al (2005). Characterization of the ERK Homologue CpMK2 from the Chestnut Blight Fungus Cryphonectria Parasitica. *Microbiology (Reading, England)* 151, no. Pt 5: 1349–58. https://doi.org/10.1099/mic.0.27796-0.

(5) Chung, Hea-Jong et al. (2006) A Gene Encoding Phosphatidyl Inositol-Specific Phospholipase C from Cryphonectria Parasitica Modulates the Lac1 Expression. *Fungal Genetics and Biology : FG & B* 43, no. 5: 326–36. https://doi.org/10.1016/j.fgb.2005.12.009.

(6) 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.

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(9) 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.

(10) Chen Chen, Qihong Sun, Buvaneswari Narayanan, Donald L. Nuss, and Osnat Herzberg (2010) Structure of Oxalacetate Acetylhydrolase, a Virulence Factor of the Chestnut Blight Fungus, The Journal of Biological Chemistry, Vol. 285, No. 34, pp. 26685–26696, August 20, 2010

(11) Levine, Bruce (2023) TACF External Grant 2019-3: Progress report

(12) Pohl, C., Kiel, J., Driessen, A., Bovenberg, R, Nygard, Y, (2016) CRISPR/Cas9 Based Genome Editing of *Penicillium chrysogenum*, ACS Synthetic Biology, (7), 754-764
DOI: 10.1021/acssynbio.6b00082

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(14) Stauber L, Prospero S, Croll D. (2020) Comparative genomics analyses of lifestyle transitions at the origin of an invasive fungal pathogen in the genus Cryphonectria. mSphere 5:e00737-20. https://doi.org/10.1128/mSphere.00737-20.

Bruce J. Levine

BruJonLev@yahoo.com (202) 549-3187

PhD Student in Plant Science with expertise in genetic modification of the chestnut blight fungus, *Cryphonectria Parasitica* (Cp).

Experience:

August 2016 to present**: Masters and Ph.D. Student in Plant Science, Institute of Bioscience and Biotechnology Research, University of Maryland, College Park**, Research focus on plant interactions with fungal pathogens. Masters awarded in June 2019. (Reference contact: Dr. Shunyuan Xiao, University of Maryland, xiao@ibbr.emd.edu .)

- Developed modified strains of Cp expressing the Cas9 endonuclease gene to improve the efficiency of genetic modification of this fungus.
- Developed monokaryon knockout strains for two genes suspected of playing a role in pathogenicity in Cp, as well as a heterokaryon strain missing a third gene, from which a monokaryon culture could not be isolate.
- Developed a reliable small-stem assay method to test the virulence of Cp strains in live chestnut seedlings during their first year of growth,
- Created gene-silencing transgenic Arabidopsis plants using RNA interference, to disrupt fungal genes via host-induced gene silencing,

1995 to present**: Volunteer and (currently) Vic President, Maryland Chapter of the American Chestnut Foundation**

- Currently serving as Vice President of the Maryland Chapter of the American Chestnut Foundation.
- Currently serving as member of the Board of the American Chestnut Foundation national organization, Chairman of its Chapters Committee, and as a member of its Science and Technology Committee.

May 1990- March 2016: **Foreign Service Officer specializing in Economics and Trade, U.S. Department of State.** Retired in March 2016 as GS15-equivalent, from a managerial position as Office Director, in which I directly supervised two employees and indirectly supervised ten. Served in various capacities at U.S. missions overseas in Taiwan, Singapore, Cambodia, China and France, as well as several Washington-based assignments.

Education:

M.S. in Plant Science, University of Maryland, College Park, awarded June 2019. Masters' thesis concerned the characterization of suspected virulence-related genes in the chestnut blight fungus, with a view to using them as targets for novel forms of disease resistance in genetically engineered plants.

B.A. in East Asian Studies, Wesleyan University, Middletown, CT, awarded 1986.

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. 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.

Translations:

"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, Advances 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, Act Pythopathologica Sinica, Vol. 26, No. 3, 1996