a. Project Title:

"Speed Breeding Darling58 and DarWin Seedlings for Pollen & Nut Production"

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b. Summary (100 words):

In 2023, the UNE chestnut lab produced generationally-advanced T3 Darling58 pollen from 30+ seedlings representing unique crosses. It then distributed 762 pollen vials to USDA permit-holding collaborators across the native range. Additionally, the lab has been speed breeding DarWin seedlings for Summer 2024 pollen. Further, the lab is breeding D58xD58 crosses for potentially homozygous seedlings and pollen. This transgenic pollen, nut, and seedling production requires intensive effort, considerable inputs, trial-and-error expertise, and vigilance to control pests and pathogens. To support the next year of these lab advances, the PI requests funding for a part-time research assistant and for lab inputs.

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c. Principal Investigator and Institutional Affiliation:

Professor Thomas Klak, University of New England (UNE), Biddeford Maine

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d. Duration of project:

One Year: December 1, 2023 to November 30, 2024

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e. Total amount requested. Please list sources and amount of matching funding for the same project:

Requested of TACF: $10,000
New York TACF Chapter Match: $10,000
Total: $20,000

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f. Short and long-term goals of the project:

short-term goals:

- Produce, and store at -80C, Darling58 pollen, including a new generation of D58 (T4), for the 2024 breeding season.

- Produce, and store at -80C, DarWin pollen for the 2024 breeding season; this would be a new accomplishement for the UNE lab.

- Breed D58xD58 seedlings to produce fertile nuts, some of which are likely to be OxO homozygous; grow out seedlings from these crosses for longer-term performance comparisons of seedlings that are non-transgenic, D58 heterozygous, and D58 homozygous.

- Assess whether the UNE lab can produce homozygous D58 pollen using the same speed breeding methods used over the last 4 years to produce heterozygous D58 pollen.

***long-term goals*:**

- Identify the Number of OxO Copies in UNE Lab-Bred D58s through PCR

- Evaluate the survival and growth performance of various transgenic chestnuts compared to each other and to their full-sibling non-OxO counterparts; collect evidence both in the lab and after outplanting in the field.

- Assess the degree to which there are significant survival and growth performance impacts from the secondary metabolism associated from the Oxalate Oxidase defense (Sengbusch 2004).

- Compare survival and growth performance of D58 non-hybrid *dentata* chestnuts and *sativa* hybrids, the latter of which have exhibited hybrid vigor; collect evidence both in the lab and in permitted orchards after outplanting them in the field (McKown and Guy 2018).

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g. Narrative (no more than five (5) pages):

Recent UNE Lab Accomplishments and How the Proposed Project Carries Them Forward to Reach New Goals:

The proposed project builds on continued speed breeding advances of the UNE lab over recent years. The lab first produced D58 pollen in late 2019. We followed, and since have enhanced, a D58 pollen production and storage protocol developed at ESF (Pilkey 2021). The UNE lab has secured -80C freezer space for D58 pollen and has then overnight-mailed pollen to USDA permit holders during the last four breeding seasons. Each year, the lab has been able to increase its quantity and genetic/geographic diversity of D58 pollen. A key factor behind the growing success has been our increasing knowledge of pest and pathogen management (Klak & May 2021). However, it is unlikely that a small indoor space that grows chestnut seedlings non-stop year-round at high density can ever be pest free. Nonetheless, through trial-and-error, intensive effort, and expert consultations, the UNE lab has been able to keep more plants alive and relatively healthy over longer time periods so that they can produce D58 pollen in quantity. Through the proposed grant, the UNE lab will be able to continue to innovate its transgenic pollen production for the 2024 breeding season and beyond.

The following paragraphs aim to establish the multi-year trajectory the UNE lab is on as it continues to make speed breeding breakthroughs. The on-going projects described below will require many hours of diligent lab work over the next year, and thereby serve to justify the hiring of a research assistant to assist the PI to carry them out.

Over the last four years, the UNE lab produced, inventoried, and stored at -80C more than three thousand vials (containing 4 microscope slides each) of genetically-diverse Darling58 American chestnut pollen. During the last year, the UNE lab produced pollen from a variety of OxO-positive nuts it received in December 2021 that were bred with wild-type trees in orchards in Meadowview VA, Lesesne State Forest VA, and ESF (NY). These sources were complemented by D58 pollen-producing nuts from wild-type trees in Maine locations. The lab continues to produce D58 pollen year-round, for the purpose of both D58xD58 lab breeding, and for the 2024 outdoor season. We continue to diversify the D58 seedlings genetically/geographically that we select for speed breeding.

In June 2023, the UNE lab overnighted 762 vials D58 pollen to USDA permit holders throughout the native range. Our D58 pollen fulfilled the quantities requested by collaborators in Georgia (Jamie Van Clief and Dr. John French), South Carolina (Dr. Joe James), Virginia (Eric Jenkins, Cassie Stark, and Dr. John Scrivani), New York (Hannah Pilkey and Allen Nichols), Pennsylvania (Stephen Hoy and Sara Fitzsimmons, and Vermont (Kendra Collins). The UNE lab provided the majority of the Darling58 pollen used this year for pollinating chestnut trees at all permitted sites and was the singular source of generationally-advanced T3 pollen used by the above permit holders. During the proposed grant period, the UNE lab aims to continue to produce advanced generations of D58 pollen to the benefit of chestnut breeding projects throughout the native range. Our lab's D58 generational advances has been reducing the timeline for reaching the T5 crosses that modelling suggests would be sufficiently genetically diverse for restoration (Westbrook et al. 2020). Indeed, the UNE lab is currently speed breeding some T5 seedlings in hopes of producing pollen for summer 2024. The lab would greatly benefit from a research assistant to help to reach the T5 pollen breakthrough.

In recent months the UNE lab has been able to make another breakthrough regarding the indoor sexual maturation of D58 seedlings (which have by now grown into saplings because they are over three-feet tall). We have been able to breed and produce fertile D58xD58 burs indoors. In late 2022, the first two dozen of these burs we harvested prior to full term and sent them to ESF for embryo rescue and tissue

culture by Hannah Pilkey. A recent result is the first PCR-documented homozygous D58 plants. These are being speed bred at ESF in hopes of homozygous pollen in 2024 or 2025.

Figure 1: Female flowers on an approx. 1.25 year old D58 sapling in the UNE lab; we have been pollinating saplings like this with pollen from other D58 saplings (note pollen on style tips); some of the offspring will inherit two copies of the OxO transgene.

During the last six months, the UNE lab has continued to speed breed Darling58 moms (that are bearing female flowers) with Darling58 dads (lab-produced pollen; see Figure 1 above); we have been able to make dozens of these crosses and advance them to fertile nut harvesting stage (Figure 2 below). Then, after stratifying the nuts and allowing them to begin to grow roots, we have begun to sow them in the lab. Some of the resulting seedlings will have inherited 2 copies of the Darling58 gene. During the grant period, we hope to genomically test those D58xD58 seedlings to ID which have no, one, and two OxO copies near the end of the 7th chromosome (see genomics discussion below). We are using speed breeding in hopes of producing pollen from the homozygous seedlings for application in the field in the summer 2024; this will mean that <u>all</u> fertile nuts produced will inherit the gene for fungal blight tolerance.

Figure 2: D58xD58 nuts that are cracking open their burs and are ready to harvest; some of these nuts should inherit two copies of the OxO transgene.

The 2023 breeding season in northern New England was severely hampered by a deep and late freeze the night of May 17-18. Female flowers did not develop on most saplings and young trees, including those at USDA permitted sites in Maine that had flowered during previous summers. This substantially reduced field pollinations with D58 pollen from the UNE lab. However, we were still able to pollinate some wild-type trees (mostly larger trees that were hardier against the freeze). These trees are located at 3 dispersed and permitted Maine locations. We pollinated with both D58 and DarWin pollen. We placed approximately 550 controlled-pollination bags, each containing on average more than 2 flowers per bag. During the winter 2023-24 portion of the grant period, the UNE lab will use the now standard histochemical assay to test for the inheritance of the OxO enzyme. However, this time we will use the bone marrow needle method to extract a sample from the cotyledon. This will reduce the damage to the nut compared to the slice method, which we have used for years but which exposes more cotyledon tissue to potential pathogen damage. The work with needles will increase the required labor hours compared to previous years. The addition of a research assistant will be valuable, particularly because undergraduates are not on campus for much of OxO-assay period in December and January.

Outplantings that will require Monitoring, Maintenance including Weeding, and Measurements during Grant Period

D58 and DarWin seedlings grown in the UNE lab during Spring 2023 were outplanted at three permitted locations in Maine for comparative study. These will require continued field work activity for which the proposed research assistant will be deployed. Here are additional details regarding our transgenic outplantings:

We continue to maintain a “common garden experiment” orchard that was first planted in 2021 and is parallel to a planting at ESF. This orchard in Cape Elizabeth, Maine contains more than 500 trees, most of which have inherited to the OxO gene; it also contains various control chestnut seedlings/saplings. We have had significant seedling loss over the past three years due to several factors including rodent predation and a 2022 summer drought (despite our distributing literally thousands of gallons of water brought to the site in 300-gallon totes). The May 17-18, 2023 hard freeze also killed a significant number of seedlings; we are studying/measuring seedling survival relative to leaf loss and ancestry, and plan to make additional measurements later in 2023. Also in 2023, we outplanted an additional 40 Darling58 and DarWin seedlings in selected spaces where the previous seedling had died. This orchard requires regular maintenance and monitoring for which a research assistant would be invaluable.

In addition, we outplanted New England’s first silviculture trial of Darling58/DarWin seedlings along with pure American controls. Approximately 80 plants were outplanted in three randomized layouts at a permitted forest restoration setting in Cape Elizabeth, Maine. Part of this experiment is to use an innovative deer repellent from Trico to replace our traditional protection from fencing or Plantra tubes where there is high deer density. Again, the proposal research assistant will be helpful in monitoring this and other transgenic chestnut sites which, notably, is a requirement of USDA/APHIS conditions.

Lastly, we outplanted another field trial comparison of 44 lab-grown plants comprised of Darling58, DarWin, and non-transgenic controls at another permitted orchard site in Phippsburg, Maine. This is the furthest north that chestnuts that carry the OxO gene have been outplanted. It is thus in part a field test of cold tolerance (Noah et al. 2021). Some of these chestnuts were outplanted as saplings that grew in the UNE lab for a year and a half and produced D58 pollen indoors. Therefore, an additional aspect of this experimental planting is to observe these saplings that were sexually-mature indoors to see if they will continue to produce pollen during the 2024 summer breeding season (if so, the catkins will require

controlled-pollination bagging per our USDA/APHIS permit). If these saplings will continue to produce D58 pollen outdoors at two and a half years of age, it could represent a breakthrough for producing sexually mature orchard trees in short order (Westbrook et al. 2020). During the grant period, the UNE lab will continue to speed breed similar D58 saplings indoors for outplanting in 2024. Research assistant help would be welcomed.

Genomics Goal: Identify the Number of OxO Copies in UNE Lab-Bred D58s through PCR

A longer-term goal of this project is to produce quantities of chestnuts that are potentially homozygous for OxO. A related goal is to be able to detect the number and genomic location of copies of the OxO transgene accurately and efficiently in any particular plant. To this end, the UNE lab is coordinating with labs at the University of Maine-Orono (Professor Ek Han Tan) and at ESF (Dakota Mathews). We would like to develop robust molecular markers to perform genetic analysis on the inheritance of the OxO transgene from Darling58, and by further extension, DarWin chestnuts that we are attempting to lab-breed for the future.

In order to ascertain the number of copies of the OxO transgene, a robust PCR-based genotyping marker can be developed by extending from the information detailed in the USDA petition for D58 deregulation (Powell et al. 2020). Our strategy for identifying whether a tree is wild-type (WT; containing no OxO gene), heterozygous, or homozygous for OxO is illustrated in the Figure 3 below. When using our proposed PCR assay, for individuals are homozygous for OxO, we do not expect an amplification product from A+B because the homozygous insertion results in a PCR band that is too large for detection. Further, the histochemical assay we use annually to identify D58(+) nuts and seedlings has three limitations which we would like to rectify. Our standard histochemical assay:
(1) is unable to detect if the T-DNA is from D58 (as opposed to other (e.g., earlier) transgenic/Darling/DarWin lines),
(2) does not reveal the genomic location of the inserted OxO transgene, and
(3) is not able to determine the number of copies of the OxO transgene.

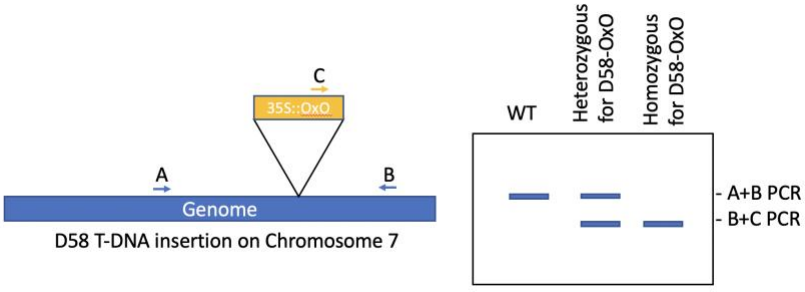

Figure 3: PCR strategy for distinguishing between trees that are wild-type (WT) containing no D58-OxO transgene, heterozygous or homozygous for D58-OxO. Amplification products from A+B are from the genome (no T-DNA) while amplification products from B+C indicates the presence of the D58-OxO inserted gene.

Based on the information the petition for deregulation, the Left border of the OxO transgene is located on Chr7:2,143,478, while the Right border is located on Chr7:2,144,082 when mapped onto the draft reference genome of *Castanea dentata* (i.e. the Ellis tree) from the Joint Genomics Institute (nd). Efforts that we have initiated aim to utilize the published primer set as well as to develop new primer sets for the OxO insertion site that will work across diverse *C. dentata* genotypes that are part of the D58 restoration breeding blocks.

If we can develop a robust PCR-based assay as described, we will be able to more accurately identify trees that carry the OxO transgene and verify its location on chromosome 7. Further, we will be able to identify homozygous OxO individuals, the pollen of which should transmit the OxO transgene to every offspring.

References:

Joint Genomics Institute. nd. "*Castanea dentata* v1.1" https://phytozome-next.jgi.doe.gov/info/Cdentata_v1_1)

Klak T, Spiers E, Powell W. 2021. "Breakthrough: Transgenic Pollen in Less Than a Year." *Chestnut: Journal of The American Chestnut Foundation*. 35(3): 27-29.

Klak T and May V. 2021. "Genetics, Transgenics, & Speed Breeding to Restore the American Chestnut." Cornell University Plant Breeding & Genetics Section Seminar Series; on-line at: https://www.youtube.com/watch?v=WqE9xY-SR2I&list=PLHPXm2Es8aQAIzSUe2l_sNQQdj8chGLrW&index=2

McKown A.D. and Guy R.D. 2018. "Hybrid vigor-poplars play it cool." *Tree Physiology*. 6(38):785-788. https://doi.org/10.1093/treephys/tpy055.

Noah PH, Cagle NL, Westbrook JW, Fitzsimmons SF. 2021. "Identifying resilient restoration targets: mapping and forecasting habitat suitability for Castanea dentata in Eastern USA under different climate-change scenarios." *Climate Change Ecology*. 2:100037. https://www.sciencedirect.com/science/article/pii/S266690052100037X

Powell W. et al. 2020. "Petition For Determination of Nonregulated Status For Blight-Tolerant Darling 58 American Chestnut" Submitted to USDA/APHIS https://www.aphis.usda.gov/brs/aphisdocs/19-309-01p.pdf

Sengbusch, PV. 2004. "The Secondary Metabolism of Plants: Secondary Defence Compounds." *Botany On-Line*. https://www1.biologie.uni-hamburg.de/b-online/e20/20.htm

Westbrook J, Holliday J, Newhouse A, and Powell W. 2020. "A plan to diversify a transgenic blight-tolerant American chestnut population with citizen science." *Plants, People, Planet*. 2, 1, 84-95.

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h. Timeline, showing start and completion dates for each goal:

Timelines for short-term goals:

- *Begun in 2019 and will continue through 2024*: Produce and store at -80C Darling58 pollen, including new generations of D58 (T4 & some T5), for the 2024 breeding season.

- *Will be started and completed in 2024*: Produce and store at -80C DarWin pollen for the 2024 breeding season; this would be new for the UNE lab.

- *Begun in 2023 and will continue through 2024*: Breed D58xD58 seedlings to produce fertile nuts, some of which are likely to be OxO homozygous; grow out all seedlings from these crosses for longer-term performance comparisons of non-transgenic, heterozygous, and homozygous chestnuts.

- *Begun in 2023 and will continue through 2024*: Assess whether the UNE lab can produce homozygous D58 pollen using the same speed breeding methods used over the last 4 years to produce heterozygous D58 pollen.

***Timelines for long-term goals*:**

- *Begun in late 2023 and will continue through 2024*: Identify the number of OxO transgene copies in UNE Lab-Bred D58s through PCR

- *Begun in 2023 and will continue through 2024 and beyond as saplings mature*: Evaluate the survival and growth performance of various transgenic chestnuts compared to each other and to their full-sibling counterparts; collect evidence both in the lab and after outplanting in the field.

- *Begun in 2023 and will continue through 2024 and beyond as saplings mature*: Assess the degree to which there are significant survival and growth performance impacts from the secondary metabolism associated from the Oxalate Oxidase transgene defense (Sengbusch 2004).

- *Begun in 2023 and will continue through to 2024 and beyond as saplings mature*: Compare survival and growth performance of D58 non-hybrid *dentata* chestnuts and *sativa* hybrids that have exhibited hybrid vigor; collect evidence both in the lab and in permitted orchards after outplanting them in the field (McKown and Guy 2018).

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i. How results will be measured and reported:

To be measured:

- Number of DarWin seedlings that produce catkins/pollen.
- Number of D58 and DarWin pollen vials from each unique cross produced.
- Regarding potentially homozygous seedlings: the number of non-OxO, heterozygous, and homozygous seedlings compared to the predicted distribution from the Punnett table.
- Quantity of homozygous Darling58 pollen produced.

To be reported:

As in the past, results will be reported in the *Chestnut Journal* or an appropriate peer-reviewed outlet.

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j. Breakdown of how and when funds will be spent:

Funding will mainly pay for a part-time research assistant hired through the University of New England Dec ’23-Nov ’24; costs are as follows:

$20 (+$6 fringe (30% of wage requirement)) x 50 weeks x 15 hrs per week = $19,500

The remaining $500 will pay for lab supplies during the grant period Dec ’23-Nov ’24.

Justification for funding to hire a part-time research assistant:

To date, the PI has singularly run the UNE transgenic chestnut lab with irregular help from undergraduate students. Academic scheduling means that undergrads are only available to assist about 6 out of 12 months; academic breaks make them unavailable half of the time. Additionally, the PI has needed to train new cohorts of undergrads each semester to do basic tasks and under supervision. The PI has been able to deploy part-time worker assistants during the summer 2023 paid through small donor contributions and grant funds (see below), but again this is short-term and seasonal. All the while, lab tasks have become more complex in the form of breeding selected crosses, continual temperature controls and adjustments due to the heat produced from additional speed-breeding lights, on-going pest and pathogen monitoring

and control, seedling sowing and management, watering and fertilizing every 48 hours, various assays, and D58 pollen collection, desiccation, -80C storage, and inventorying.

There is thus an urgent need to hire a person to provide longer-term and consistent weekly work throughout the calendar year. The proposed 15 hour per week schedule for the research assistant would mean the person could work 3 days per week for 5 hours. This would go a long way to complementing the PI's lab efforts and allow him more time to address the overarching issues associated with leading and continually seeking and securing funding for a successful transgenic speed breeding lab and outplanted orchards and silviculture trials. The proposed part-time research assistant hire can be seen as a one-year test run; if it proves to be successful, the PI will seek additional funding from donors and grants to pay a research assistant after the proposed grant period, potentially for more than 15 hours per week.

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k. Brief Curriculum Vitae (CV) for each Principal Investigator, including recent publications and grants received. Please restrict each CV to two (2) pages:

THOMAS KLAK

tklak@une.edu; 207-391-9496
2011- present: Professor, School of Marine & Environmental Programs, University of New England, Biddeford Maine

A. Education:

Ph.D., 1987, Geography, University of Wisconsin-Madison
M.S., 1982, Geography, University of Wisconsin-Madison
B.A., 1979, Augustana College, Rock Island, IL

B. Published Articles on Chestnuts:

2021. Thomas Klak, Ellen Spiers, and William Powell. 2021. "Breakthrough: Transgenic Pollen in Less Than a Year" *Chestnut: The Journal of The American Chestnut Foundation* Vol. 35, No. 3 (Fall), pp.27-29.

2021. Thomas Klak and Vernon Coffey, "Is Controlled Pollination Really Controlled?" *Chestnut: The Journal of the American Chestnut Foundation,* Vol. 35, No. 2, pp. 31-33.

2020. Thomas Klak, Woon Yuen Koh, Tyler Riendeau, and Andrew Grammas, "Comparison of Four Methods for Non-Destructive Testing of Chestnut Seeds for OxO Gene Activity" *Chestnut: The Journal of the American Chestnut Foundation,* Vol. 34, No. 3, pp. 31-34.

2018. Thomas Klak and Matthew Sutherland, "Planting Pure American Chestnuts on Wild and Remote Long Island, Maine" *Chestnut: The Journal of the American Chestnut Foundation,* Vol. 31, No. 3, pp. 12-14

2018. Thomas Klak, "Preserving and Making Accessible American Chestnut Biodiversity in Maine" *Chestnut: The Journal of the American Chestnut Foundation* Winter, 32(2)10-12

2018. Thomas Klak, "TACF-Landowner Partnerships: An Unfolding Story from Maine" *Chestnut: The Journal of the American Chestnut Foundation* Fall, 32(3)10-12

C. External Transgenic Chestnut Grants for which I was PI:

2022. $6,000 from the PW Sprague Memorial Foundation to support transgenic chestnut fieldwork in Cape Elizabeth, ME.

2021. $10,000 from the Cricket Island Foundation.

2021. $7,500 from the PW Sprague Memorial Foundation to support chestnut fieldwork on a Common Garden Experiment involving transgenic chestnut seedlings.

2019. $10,000 from The American Chestnut Foundation External Grants “Comparison of Alternative Speed Breeding Methods to Produce Chestnut Pollen”.

2019. $12,500 PI. from the Quimby Family Foundation “Blight-Tolerant American Chestnut Restoration on Protected Maine Lands”.

2019. $5,000 from the PW Sprague Memorial Foundation to support chestnut fieldwork on the Common Garden Experiment.

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l. A Conflict of Interest or Commitment (COI or COC) statement*:* There are no conflicts of interest.