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PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR BLIGHT-TOLERANT DARLING 58 AMERICAN CHESTNUT

(Castanea dentata)

Submitted January 17, 2020

Event name: Darling 58 and offspring

Tentative OECD Unique Identifier: ESF-DAR58-3

No CBI. Unfavorable information: None.

The undersigned submits this petition under 7 CFR 340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR part 340.

Submitted by



William A. Powell, Ph.D.

Director, American Chestnut Research and Restoration Project

State University of New York College of Environmental Science and Forestry

1 Forestry Drive, Syracuse NY, 13210, 315-470-6744

wapowell@esf.edu



State University of New York College of
Environmental Science and Forestry

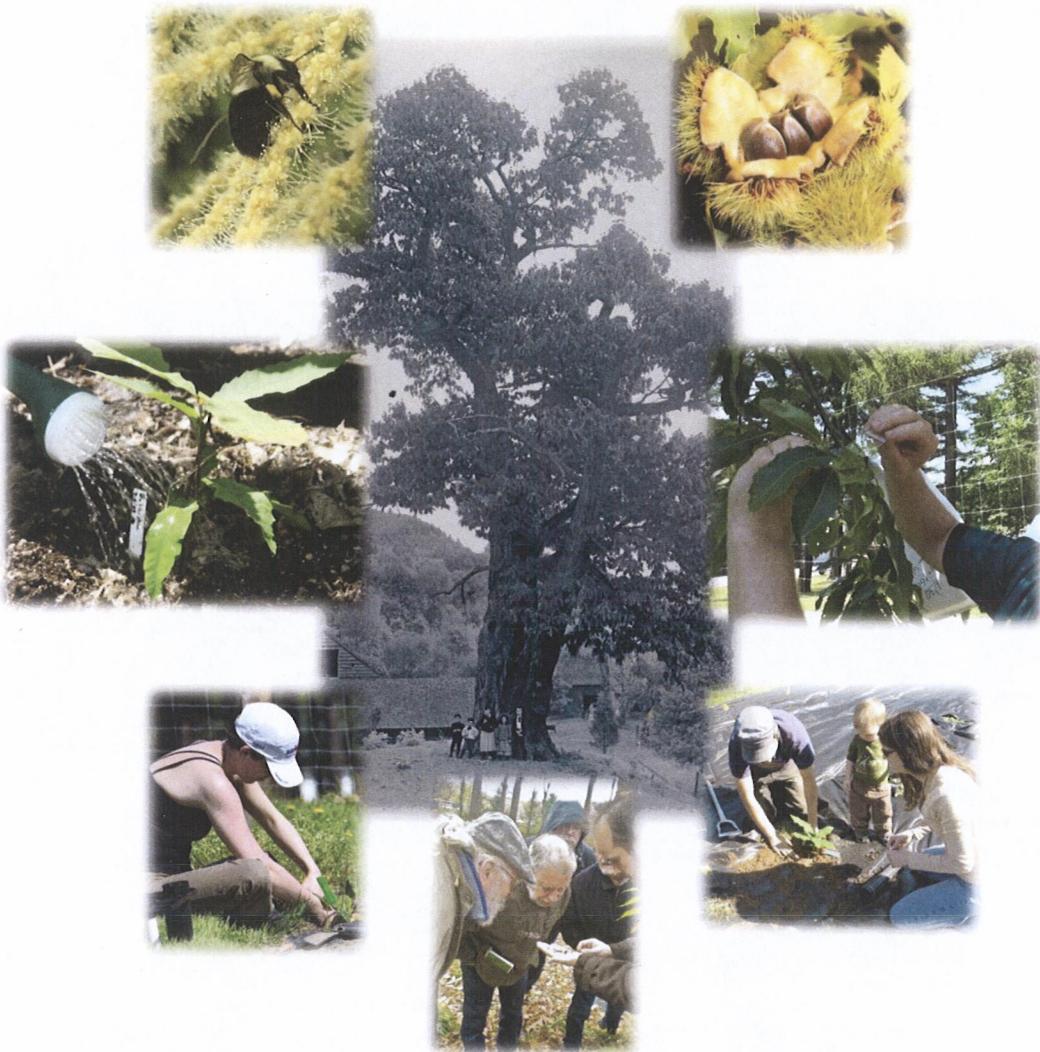
Prepared by

Andrew Newhouse, Vernon Coffey, Linda McGuigan, Allison Oakes, Kaitlin Breda, Dakota Matthews, John Drake, John Dougherty, John French, Michael Braverman, Charles Maynard,
William Powell

Contributors

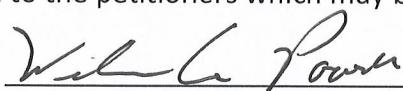
A. Allwine, K. Baier, A. Brown, N. Cannon, E. Carlson, J. Carlson, K. D'Amico, T. Desmarais, C. Duong, S. Garrett, J. Gibbs, H. Goldspiel, A. Gray, T. Horton, J. Johnson, D. Leopold, S. McArt, L. Northern, A. Onwumelu, H. Pilkey, H. Roden, J. Rushton, K. Russell, E. Saraiva, S. Satchwell, M. Staton, S. Stehman, B. Sweeney, A. Teller, T. Tschaplinski, L. Will, J. Zarnowski, B. Zhang

Darling 58 Blight-tolerant American Chestnut for Restoration and Forest Health



Certification

The undersigned certify that, to the best of their knowledge and belief, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioners which may be unfavorable to the petition.



William A. Powell, Ph.D.

State University of New York College of Environmental Science and Forestry
1 Forestry Drive, Syracuse NY, 13210
315-470-6744

Executive Summary

Researchers at the State University of New York College of Environmental Science and Forestry (ESF) have developed Darling 58 American chestnut (*Castanea dentata*) trees with enhanced blight tolerance. This enhanced blight tolerance trait is generated by a single gene and can be passed on to subsequent generations through classical Mendelian inheritance. The purpose of these trees is not to replace the surviving remnant American chestnut population, but to help rescue it by allowing introgression of the blight tolerance trait and to ultimately produce a viable and diverse restoration population from their offspring. Because offspring of Darling 58 trees will include both transgenic and non-transgenic individuals, the original wild-type American chestnut will be conserved far into the future.

To our knowledge this is the first petition for a bioengineered organism with the goal of ecological restoration, and represents a unique application for this technology to be potentially used for environmental and cultural benefits outside agriculture. This petition requests that the bioengineered Darling 58 event of American chestnut (and its offspring) be granted nonregulated status by APHIS because it does not pose a plant pest risk as compared to its isogenic controls or traditionally bred chestnuts. Therefore, it should no longer be considered a regulated article under 7 CFR Part 340.

The American chestnut was once one of the most abundant trees within its range in the eastern United States. It was a fast-growing and long-lived canopy tree that produced a consistent crop of healthful nuts, could be harvested for valuable lumber, and was considered a keystone species for wildlife. That ended when an invasive fungal pathogen, *Cryphonectria parasitica*, was introduced from Asia and killed over 3 billion American chestnuts throughout their natural range.

Tolerance to this exotic pathogen in Darling 58 American chestnuts was enhanced by adding a gene for an enzyme called oxalate oxidase (OxO). This enzyme has no direct fungicidal properties, but rather detoxifies oxalic acid (oxalate) produced by the fungus, preventing the acid from killing the chestnut's tissues which can lead to lethal cankers on the tree. In the presence of OxO, the damage caused by the oxalate is significantly restricted, resulting in superficial cankers. For this reason, the tree can coexist with the fungus in a manner similar to Asian chestnut species in the fungus' natural range. Tolerance describes a plant defense mechanism that does not involve direct pesticidal mechanisms, but rather allows plants to survive and reproduce despite pathogen infections. Tolerance mechanisms without pesticidal activity tend to reduce selective pressures that might otherwise allow a pathogen to overcome a plant's defense. Consequently, tolerance mechanisms are generally more stable and sustainable than other types of resistance, which reduces plant pest risks related to the durability of the defense or adaptations by the pathogen. This also means these trees will not require forest management interventions such as planting refugia or other practices that are sometimes used to maintain a plant defense mechanism.

Oxalate oxidase is a common enzyme found in all grains, several other crops and food products, and many wild plants and microbes. OxO and other enzymes that detoxify oxalate function as natural defenses against the effects of specific pathogens that produce oxalic acid. OxO is well understood and has been studied for over 100 years. There are even functionally similar genes in Chinese chestnuts, which may partially contribute to the blight tolerance observed in these trees.

We specifically chose an OxO gene from wheat because it is well characterized, effectively detoxifies oxalate, and is consumed daily by people and livestock. Although it is from wheat, OxO is not related to gluten and does not match any known allergens from wheat or other sources. Independent nutrition analyses have confirmed that transgenic chestnuts are not nutritionally different than their wild-type relatives. Even with the ubiquity of OxO in the environment and agriculture, there are no reports of this enzyme being detrimental to human or animal health, having adverse effects on the environment, or being a plant pest risk.

Darling 58 American chestnuts have a single insertion of two genes added to the over 30,000 gene pairs in the chestnut genome. Based on genomic analysis, the insertion does not disrupt any known gene. In addition to the gene for OxO described above, a selectable marker called neomycin phosphotransferase (NPTII) was added for use in the development of these trees. The NPTII gene has been repeatedly evaluated for safety and is found in many bioengineered plants with nonregulated status or exemptions from the USDA, EPA, and FDA. Although many wild and cultivated plants have been found to naturally contain *Agrobacterium* sequences, no additional *Agrobacterium tumefaciens* vector sequences are present in Darling 58 that might present plant pest risks. Darling 58 American chestnuts retain 100% of their natural complement of genes; no native genes or alleles have been removed or replaced, and expression of nearby genes is not affected.

Several experiments have been performed on OxO-expressing American chestnuts, and results consistently confirm a lack of plant pest risks or non-target effects. Studies have been conducted on Darling 58, offspring of Darling 58, and on older legacy events that also express OxO. These experiments included observing mycorrhizal colonization of chestnut roots, aquatic and terrestrial insect herbivory on leaves, wood frog tadpoles feeding on leaf litter, leaf litter decomposition, interactions with nearby plants, and use by bumble bees of OxO-containing chestnut pollen. Nutritional composition and tannin concentrations of the OxO-containing nuts have been evaluated by commercial testing labs, and the OxO enzyme was queried against allergen, gluten, and toxin databases. In all cases, the blight-tolerant transgenic American chestnut trees were shown to be equivalent to wild-type American or traditionally bred hybrid chestnuts.

First-generation (T1) offspring of Darling 58 have not shown any growth differences due to transgene presence after two growing seasons. Second-generation (T2) offspring have been generated from several additional parental crosses, some of which appear to show slower first-season growth of transgenic compared to non-transgenic seedlings, while other crosses show no growth differences due to OxO presence. Other chestnut studies have shown that first-year mid-season height measurements do not consistently predict long-term growth trends, so growth of these T2 offspring will be closely monitored in coming seasons. In crosses where T2 growth differences were detected, they were smaller than natural tree-to-tree differences among wild-type chestnuts. No significant differences have been observed in terms of plant pest risk traits such as competitiveness, responses to other pests, interactions with other organisms in the environment, or survival (besides blight tolerance). Therefore, Darling 58 American chestnuts should present no additional weediness traits or plant pest risks than wild-type American chestnuts or traditionally bred hybrids. The American chestnut is not considered an invasive, fast-colonizing tree, and the OxO gene will not change these traits.

If Darling 58 American chestnuts are granted nonregulated status, they will be made available for not-for-profit distribution to the public, and to groups including private, indigenous, state, and federal restoration programs, depending on the goals and preferences of these various groups. Initial distribution will consist of long-term research plots and relatively small-scale public horticultural plantings, both of which will be monitored with the help of citizen scientists and will inform subsequent larger-scale distributions. Restoration efforts will primarily be managed by The American Chestnut Foundation (TACF), a non-profit organization which is a supporter and collaborative partner with ESF.

ESF's research program was initiated by public chestnut enthusiasts who became founding members of the New York Chapter of TACF, and the vast majority of ESF's research funding has come from public, government, philanthropic, and other non-corporate sources. Following the spirit of transparency and public interest in chestnuts, Darling 58 trees are not patented, so as not to impede any American chestnut distribution or restoration efforts. Researchers will continually seek feedback, but the public will ultimately be able to propagate these trees, share them, and plant them as they wish.

One benefit of this type of distribution is engagement from citizen scientists who wish to help with the restoration of this species. TACF and ESF are developing a plan to cross Darling 58 with a diverse set of surviving American chestnuts over multiple generations, which should result in a diverse and resilient population suitable for potential large-scale restoration efforts. This is part of a broader restoration effort including complementary approaches such as backcross breeding and biocontrol treatments, as well as managing other threats like *Phytophthora* root rot. Regardless of the methods used, meaningful restoration will require patience and dedication, because American chestnuts, compared to other hardwood tree species within their natural range, are relatively slow to spread to new areas. Therefore, efforts toward outcrossing with wild chestnuts and the resulting increase in genetic diversity will rely on the public to restore this keystone species to our forests.

Successful colonization by transgenic chestnuts in areas beyond where they are intentionally planted will be relatively slow and manageable, depending on the preferences of land managers. Managing unwanted pollination of chestnut orchards is already an issue that is addressed by chestnut growers, since pollen from certain hybrid or interspecific crosses can be detrimental to harvests. Small effective pollination distances for chestnut mean that such management is easily achievable. Controlling pollination by transgenic chestnuts after implementation of potential restoration programs would be similarly manageable for growers if needed.

Since Darling 58 trees do not pose novel plant pest risks, they should be granted nonregulated status so they can be distributed and planted like wild-type or traditionally bred chestnuts to accomplish meaningful conservation and restoration of the American chestnut.

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Nomenclature, Terminology, and Usage

Event **Darling 58** was initially called LM-B4SX58 for laboratory record-keeping. Abbreviations used in figures, other documents, or publications have included D58, Dar58, and SX58. We have attempted to use the name Darling 58 exclusively in this document, but secondary sources or other references may use these other names to refer to the same event. Additionally, transgenic offspring lines (T1 and T2 generations; see Section 6.4) have been given names starting with “D58+...” to identify the transgenic parent and positive confirmation of transgene presence; these are explained in this petition where such abbreviations are used.

Darling 58 belongs to Phenotypic Designation “Chestnut Blight Resistant / p35S-OxO” as noted in USDA-APHIS notifications and permits (see Appendix I).

“ESF-DAR58-3” has been tentatively designated as an OECD Unique Identifier for Darling 58.

Darling 58 has recently been listed as a cultivar (called ‘Darling’) by the Connecticut Agricultural Experiment Station (Anagnostakis, 2019), but for the purposes of this petition, we will refer to it as a transgenic event rather than a cultivar.

The isogenic line (genetic background) of Darling 58 is known as **Ellis**. This line was derived from a seed borne on a wild-type American chestnut tree found near Binghamton, New York by members of The American Chestnut Foundation (Section 6.1).

When referring to pollination and crosses for which both parents are known, the female parent is listed first, followed by the male parent or pollen source (i.e., Female parent x Male parent).

An original individual plant from which clonal propagules have been produced is known as the “ortet”, while clonally propagated individuals can be called “ramets” (to distinguish them from ortets) or “plantlets” (to distinguish them from plants grown from seed).

See Section 10 for information and nomenclature regarding older “legacy” transgenic chestnut events described for background context, comparisons, or bridging data. Bridging refers to the use of data from other transgenic events intended to support conclusions on a primary event.

All transgenic events described in this document were transformed using *Agrobacterium tumefaciens*. However, in keeping with modern scientific usage, we typically refer to this vector agent and related techniques using the name “*Agrobacterium*” without the specific epithet.

The State University of New York College of Environmental Science and Forestry can be referred to as SUNY-ESF, or by the preferred singular acronym ESF.

The American Chestnut Research & Restoration Project (informally called the “chestnut project”) at ESF was formally established by the New York State Senate in 1997 (State of New York, 1997). ESF’s chestnut project is a non-profit initiative with the support and close collaboration of The American Chestnut Foundation (TACF; a non-profit 501(c)(3) charitable organization).

Abbreviations and Definitions

| | |
|-------------------------|--|
| 35S | CaMV-35S promoter |
| AA | Amino acid |
| AC | American chestnut |
| Act2 | Actin2 terminator regulatory sequence |
| ANOVA | Analysis of variance |
| APHIS | Animal and Plant Health Inspection Service |
| B3F3 (BC3F3) | Third-generation backcross chestnut line |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pairs |
| CC | Chinese chestnut |
| CD | Celiac disease |
| cDNA | Complementary DNA (synthesized from RNA) |
| CFR | Code of Federal Regulations |
| D4 | Darling 4 (transgenic event) |
| D54, D58 | Darling 54, Darling 58 (transgenic events) |
| DBH | Diameter at breast height |
| $\Delta\Delta\text{ct}$ | Delta-delta cycle threshold (qPCR analysis method) |
| DNA | Deoxyribonucleic acid |
| DPI | Days post inoculation |
| E1 | Embryo initiation medium 1 |
| EC | Enzyme Commission number |
| EM | Ectomycorrhizal |
| EP155 | Strain name of <i>Cryphonectria parasitica</i> (ATCC38755) |
| EPA | United States Environmental Protection Agency |
| ESF | State University of New York College of Environmental Science and Forestry |
| F1 | Filial 1, first offspring generation from hybrid cross |
| FASTA | Fast Alignment (DNA and protein sequence alignment software) |
| FDA | United States Food and Drug Administration |
| FIA | Forest Inventory and Analysis |
| FIFRA | Federal Insecticide, Fungicide, and Rodenticide Act |
| GFP | Green Fluorescent protein |
| GLP | Germin-like protein |
| GMO | Genetically modified organism |
| GOI | Gene of interest |
| HSD | Honestly significant difference (Tukey's range test, a statistical comparison) |
| HY | Hybrid |
| ID | Identity |
| IKB | Internal kernel breakdown |
| LCP | Light compensation point |
| LSP | Light saturation point |
| mRNA | Messenger RNA |
| NASEM | National Academies of Science, Engineering, and Medicine |

| | |
|---------|--|
| NCBI | National Center for Biotechnology Information (ncbi.nlm.nih.gov) |
| NCSU | North Carolina State University |
| NPT | Neomycin phosphotransferase |
| NT | Non-transgenic |
| NY-TACF | New York Chapter of The American Chestnut Foundation |
| OA | Oxalic acid |
| OP | Open pollinated |
| OTU | Operational taxonomic unit |
| PCR | Polymerase chain reaction |
| PPFD | Photosynthetic photon flux densities |
| qPCR | Quantitative (real-time) PCR |
| RDI | Recommended Daily Intake |
| RFLP | Restriction fragment length polymorphism |
| RNA | Ribonucleic acid |
| RT-qPCR | Reverse transcriptase quantitative PCR |
| SE | Standard error |
| SEM | Standard error of the mean |
| spp. | Species |
| SUNY | State University of New York |
| SX58 | Darling 58 |
| T0 | Original transgenic generation (not outcrossed or bred) |
| T1 (T2) | First (second) offspring generation from cross with a transgenic parent |
| TACF | The American Chestnut Foundation (also ACF) |
| T-DNA | Transfer DNA |
| TEM | Transmission electron microscopy |
| TG | Transgenic |
| UBQ | Ubiquitin (regulatory sequences) |
| U.S. | United States |
| USDA | United States Department of Agriculture |
| USFS | USDA Forest Service |
| VC | Vegetative compatibility |
| vspB | Vegetative storage protein B |
| WT | Wild type (noun) or wild-type (adjective); non-transgenic |

1.0 Rationale for development of Darling 58 American chestnut

1.1 Basis for the request for a determination of nonregulated status under 7 CFR § 340.6

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. § 7701-7772), to prevent the introduction and dissemination of plant pests into the U.S. APHIS regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article, pending regulatory decisions by other federal agencies.

The State University of New York College of Environmental Science and Forestry (ESF) is submitting this petition to APHIS for a determination of nonregulated status for the new biotechnology-derived, blight-tolerant American chestnut (*Castanea dentata* (Marsh.) Borkh.) tree, Darling 58, and any progeny derived from crosses between Darling 58 and any sexually compatible *Castanea* species. As described in this petition, Darling 58 has been studied in detail and no plant pest or environmental risks have been observed. Transgene presence has repeatedly been shown to result in fewer changes than traditional breeding in other plants (Schnell *et al.*, 2015; Anderson *et al.*, 2016; Herman *et al.*, 2017), and we have found that Darling 58 American chestnut is following the same trend. The *Agrobacterium*-mediated transformation process also does not necessarily increase plant pest risks, as this is a natural phenomenon which has resulted in about 7% of dicot species sequenced to date containing DNA from *Agrobacterium* (Matveeva and Otten, 2019).

1.2 American chestnut and chestnut blight

Section 2 provides detailed information on the American chestnut and other members of the genus *Castanea*; Section 3 describes the blight fungus and chestnut blight as a disease. This subsection is an overview to provide general context for this petition.

The American chestnut is a large, deciduous tree native to eastern North America. Before the introduction of chestnut blight, American chestnut had a range that extended across eastern North America from Mississippi to Maine, including nearly every state east of the Mississippi River as well as southern Ontario, and was found at every elevation from sea level to over 5000 ft. In many areas, especially on mountain slopes in the Appalachian range, American chestnut was a dominant forest tree and a keystone species.

American chestnut provided a consistent nut crop that was consumed by numerous mammals, birds, and insects. The nuts were important both ecologically and agriculturally, as they were consumed by people and livestock. Chestnut wood and leaf litter influenced ecosystem structure and function in areas where it was a dominant tree (Ellison *et al.*, 2005).

Chestnut wood is light, rot resistant, and easily workable (Saucier, 1973); it was used for general construction, furniture, boxes and crates, railroad ties, pulpwood, shingles, and fuel, among other uses. It was also the primary source of tannin for the leather industry. Before the introduction of

blight, more American chestnut wood was commercially harvested in the United States than any other single species (Buttrick, 1915).

In the first decade of the twentieth century, a fungal canker disease was discovered in the Bronx Zoological Park, New York, which was disfiguring and quickly killing American chestnut trees (Merkel, 1905). The fungus was described by mycologist William Murrill (1906) as *Diaporthe parasitica*, which was soon reclassified *Endothia parasitica* (Anderson and Anderson, 1912) and later *Cryphonectria parasitica* (Murrill) Barr (herein referred to as *C. parasitica*). In the decades following its discovery, the blight would spread throughout the chestnut range and kill billions of trees. The blight fungus infects the stem via wounds or cracks in the bark. It has been shown that the pathogen kills living tissue primarily by secreting a toxin called oxalic acid (McCarroll and Thor, 1978). Early attempts were made to control the spread of the disease, including quarantine and destruction of diseased trees. These were ineffective because of the fungus' ability to produce prolific spores which spread by animals and the wind (Anagnostakis, 1987; Rigling and Prospero, 2018), as well as its ability to reproduce as a saprophyte on other tree species and on dead American chestnut stems (Prospero *et al.*, 2006; Section 5.2). These factors, in conjunction with American chestnut's extreme susceptibility to infection, overwhelmed all attempts at containment (Hepting, 1974; Anagnostakis, 1987). Other tree species, mostly oak, maple and hickory, have filled the space left by dying chestnut trees (Figure 1.2a); American chestnut is now considered functionally extinct in modern forests (Ellison *et al.*, 2005).



Figure 1.2a. Example of a pure American chestnut stand a few years after blight infection. (Photo: A ghost forest of blighted American chestnuts in Virginia; Library of Congress Prints and Photographs Division¹).

No wild-type American chestnut tree with documented blight resistance has been found, but American chestnut has avoided extinction in the wild through its ability to sprout new shoots from

¹ Washington, D.C. 20540, USA. Available at: <http://www.loc.gov/pictures/item/va1798.photos.192521p/>

the root collar of diseased trees (Paillet, 2002). While the population has been reduced drastically, millions of (mostly immature) trees remain in the wild (Dalgleish *et al.*, 2016) and in orchards (Fitzsimmons, 2017), which should allow re-establishment of a viable, genetically diverse population if a heritable disease resistance or tolerance trait can be introduced (Section 11.2).

1.3 Darling 58 transgenic American chestnut

The American Chestnut Research and Restoration Project at ESF has developed the Darling 58 transgenic American chestnut (Section 6). Darling 58 shows enhanced tolerance to the fungus *C. parasitica*, typically showing blight symptoms similar to or less severe than blight-resistant Chinese chestnut (Section 8.1). Darling 58 expresses a wheat gene for oxalate oxidase (Dratewka-Kos *et al.*, 1989; Section 4) which degrades oxalic acid and protects the tree from damage caused by chestnut blight. Darling 58 and all other transgenic American chestnut events discussed in this petition (“legacy events”, Section 10) were developed using *Agrobacterium*-mediated transformation (Section 6.2). Due to their similarity to Darling 58 (i.e., transgenic American chestnut expressing OxO), some legacy events are described in this petition to provide supporting evidence or bridging data that is applicable to Darling 58 (Table 1.3a; Section 10).

Transgene inheritance (Section 6.4) and molecular characterization (Section 7.2) have demonstrated the stable integration of a single copy of OxO into a safe, non-coding region of the American chestnut genome in Darling 58. Phenotypic observations including growth and photosynthesis (Section 8) and environmental interaction experiments (Section 9) on Darling 58 have been conducted in laboratories, greenhouses, and permitted field plots (Appendix I), all of which demonstrate that Darling 58 is not significantly different from non-transgenic chestnut controls. Nutritional analysis of Darling 58 chestnuts shows that there are no substantial nutritional differences compared to non-transgenic nuts, and analysis of the OxO enzyme indicates a lack of allergenicity or toxicity (Section 8.4). Table 1.3a summarizes experiments, results, and tissue sources for data on Darling 58 and selected OxO-expressing legacy events.

Pending the decisions of APHIS and other federal agencies (Section 1.6), Darling 58 American chestnut could be publicly distributed and bred with surviving American chestnuts, allowing diverse offspring to be used in restoration plantings to reintroduce American chestnut as a self-sustaining forest tree species in its native range (Section 11.2). Forest restoration with blight-tolerant American chestnut would be an iterative process, incorporating feedback from continuing research and early small-scale releases, and enhancing genetic diversity from surviving wild American chestnuts before widespread restoration plantings are implemented (Westbrook *et al.*, 2019a). Reintroduction of blight-tolerant American chestnuts could offer unique opportunities to restore ecological relationships that have been missing from the eastern U.S. for more than a century, as well as economic and cultural opportunities. Darling 58 also could be used to breed with other *Castanea* species (Section 2.1), such as European chestnut (*Castanea sativa*) and North American chinquapin species (*C. pumila* and *C. ozarkensis*), all of which are susceptible to chestnut blight.

*Table 1.3a. Experiments performed on key OxO-expressing transgenic events, including legacy events described in this petition for comparison to Darling 58. Parenthetical number indicates petition section where data are presented; gray background indicates data not available (X) or not presented here for bridging. Sources of transgenic plants/tissues: * indicates plant grown in a growth chamber or greenhouse (i.e. not part of a release authorization); ‡ indicates plant grown under permit 17-053-103r; † indicates permit 14-022-102r; § indicates permit 10-357-118r.*

| Type of data | Event: | Darling 4 | Darling 5 | Darling 215 | Darling 54 | Darling 58 |
|--|---|--|--------------------------|--------------------------|--|------------|
| Inheritance | T1 (10.3) † | X | X | T1 (10.3) † | T1, T2 (6.4) †‡ | |
| Vector insert copies (see Table 10.1a) {analysis method} | 2 GOI, 2 GFP (10.4.1) * {Southern, qPCR} | 1 GOI, 1 GFP (10.4.1) * {Southern, qPCR} | X | 1 GOI (10.4.1) * {qPCR} | 1 GOI (7.2.2, 6.4) †‡ {qPCR; Seq. in progress} | |
| Insert location (flanking sequence) | (Analysis in progress) | X | X | Inside intron (10.4.2) * | Not near known gene (7.2.3) * | |
| OxO mRNA expression (relative to Dar. 215) | <0.1X (7.3, 10.4) † | X | 1X (7.3, 10.4.3) † | 3X (stem) (10.4.2) ‡ | 4X (stem) (7.3) ‡ | |
| OxO enzyme activity | Vascular (10.1) * | Vascular | High | High (10.4.4) * | High (7.4.1) *†‡ | |
| OxO enzyme quantification | X | X | X | 0.5-1.1 µg/mg (10.4.4) * | 0.3 - 1.1 µg/mg (7.4.2) *‡ | |
| Blight tolerance vs. Chinese (CC) | Intermed. (<CC) (10.5.1) †§ | Low (<<CC) | High (=CC) (8.1.1) * | High (≥CC) (10.5.1) * | High (≥CC) (8.1) *‡ | |
| Growth / form vs. NT | Same | Same | X | Same | Similar (8.2) †‡ | |
| Photosynthesis vs. NT | X | X | X | X | Similar (8.3) ‡ | |
| Nutrition vs. NT | Same (10.5.2) † | X | X | X | Similar (8.4.1) ‡ | |
| Mycorrhizae vs. NT | Same (10.6.1-2) † | Same (10.6.2) § | X | Same (9.1.1) * | Similar (9.1.1) * | |
| Native plant interactions | Field growth (10.6.5) § | X | X | X | Native seed germ. (9.1.2) ‡ | |
| Insect herbivory | Aquatic & terr. (10.6.6 - 7) † | X | X | X | Terrestrial, tri-trophic (9.1.3) ‡ | |
| Bumble bee use of pollen with OxO | X | X | X | X | Similar (9.1.4) (*purified enzyme) | |
| Response to other pests | No visible diff. (10.6.9) † | No visible differences † | No visible differences * | No visible differences ‡ | No visible differences (9.2) ‡ | |
| Transgenic leaf litter: decomposition, OxO persistence | Persistence, decomp. (10.6.3 - 10.6.4) †§ | X | X | X | OxO persistence (9.4) * | |
| Tadpole development and survival vs. NT | Same (10.6.8) † | X | X | X | X | |

1.4 Oxalic acid tolerance allows coexistence of the tree and the fungus

The oxalate oxidase enzyme (OxO; EC1.2.3.4) expressed by the Darling 58 American chestnut catalyzes the degradation of oxalic acid into carbon dioxide and hydrogen peroxide, which allows the Darling 58 American chestnut to protect its living tissues from damage by oxalic acid exuded by the blight fungus. Degradation of oxalic acid reduces necrosis of living tree tissues at the margins of cankers (Sections 6.3 and 8.1). This mechanism is not lethal to the fungus or inhibitory to its replication; it continues to live as a saprophyte on Darling 58 American chestnut trees as it does on Chinese chestnuts and many other tree species (Stipes *et al.*, 1978; Nash and Stambaugh, 1987; Baird, 1991; Davis *et al.*, 1997). Efficacy of Darling 58 trees tolerating blight infections has been demonstrated in both leaf and stem inoculation assays, which compare the response of Darling 58 to wild-type American chestnut and Chinese chestnut controls (Section 8).

OxO offers a mechanism specifically effective at protecting American chestnuts from the effects of the blight, without direct broad-spectrum anti-fungal properties. Since this transgene product does not act against the fungus (Section 4), but rather allows the tree to tolerate infections without fatal damage (Section 8.1), Darling 58 chestnuts are more appropriately termed blight *tolerant* than blight *resistant* (Section 6.3). This tolerance mechanism should provide a uniquely stable plant defense, as the likelihood of a pathogen evolving to overcome a tolerance trait is minimized in the absence of a strong selective pressure (Section 5.3).

1.5 Existing means of addressing chestnut blight

To date, no strategy has been effective in protecting pure American chestnut trees from the effects of chestnut blight on a landscape scale. Chemical control has not been shown to be effective in addressing chestnut blight in American chestnut (Section 3.3.4). Biocontrol methods hold promise for treating individual trees or close stands of chestnuts, but are generally not effective in controlling blight on a landscape scale in the U.S. (Section 3.3.1). Mutational breeding experiments were conducted over several years using radiation treatment of seeds (Section 3.3.3), but resulting trees have not shown enhanced blight resistance.

Hybrid breeding programs have been conducted for decades in an attempt to incorporate resistance-enhancing genes from Asian chestnut species (Section 3.3.2). The largest breeding program currently underway is the backcross breeding program conducted by The American Chestnut Foundation (TACF). Trees produced from this breeding program have shown resistance to blight at levels intermediate between susceptible American chestnut and resistant Chinese chestnut. The genotype of these trees is mostly American chestnut, with a small percentage of Chinese chestnut DNA, including both resistance-enhancing genes and other genes that have been incorporated incidentally. In contrast, the Darling 58 genome contains a complete complement of American chestnut genes, with the addition of the T-DNA region of the p35S-OxO vector containing the OxO gene for blight tolerance and a selectable marker (Section 7).

1.6 Submissions to other regulatory agencies

Under the Coordinated Framework for Regulation of Biotechnology (Office of Science and Technology Policy, 1986; Latham, 1992; McEwan *et al.*, 2006), the responsibility for regulatory oversight of biotechnology-derived plants falls primarily on one or more of three U.S. agencies:

the United States Department of Agriculture (USDA), U.S. Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA), each agency's oversight depending on different and specific criteria. Therefore, in addition to the USDA, documentation on the Darling 58 blight-tolerant American chestnut trees will be submitted to the FDA for review. The precise role of the EPA in reviewing this unique product is still being discussed.

Darling 58 blight-tolerant American chestnut trees produce edible nuts, and although these trees were developed for forest restoration and not for agricultural use, the nuts will likely be consumed by humans and livestock. Therefore, regulation of Darling 58 trees falls within the scope of the U.S. Food and Drug Administration's policy statement concerning regulation of products derived from new plant cultivars, including those developed through biotechnology (Federal Food, Drug, and Cosmetic Act, as clarified in 57 FR 22984). A food and feed safety and nutritional assessment of Darling 58 will be submitted to the FDA for review.

Oxalate oxidase (OxO) is not intended to prevent, destroy, repel, or mitigate the blight fungus, so it is different from products subject to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Nevertheless, we have briefed the EPA on the product and technology at intervals, and will share with them this petition.

In the future, we also anticipate regulatory submissions to the Canadian Food Inspection Agency (CFIA) and Health Canada. The natural range of the American chestnut extends into Canada, so Darling 58 trees may be introduced or eventually naturally introgress across the border. We note that American chestnut dispersal is relatively slow without human intervention and is quite easy to monitor and control if desired (Sections 2.2.2 and 11.6), so successful establishment of any new type of American chestnut in a new region will likely require public support coupled with intentional planting and maintenance efforts.

2.0 Biology of chestnuts

2.1 Taxonomy and distribution of chestnuts

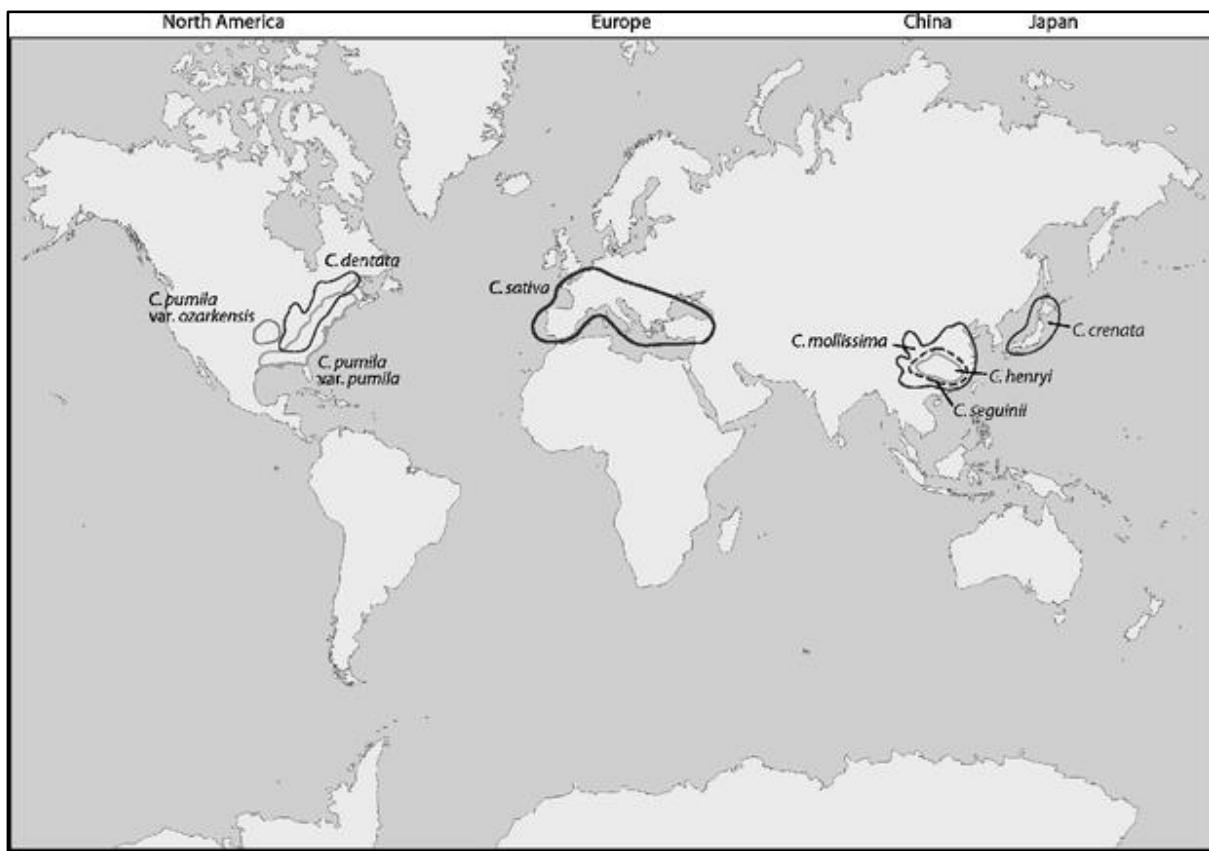
American chestnuts are a member of the genus *Castanea* in the plant family Fagaceae. Depending on the taxonomic reference, the Fagaceae family contains between 220 and 900 species worldwide in 6 to 10 genera (Kremer *et al.*, 2012; ITIS, 2019), consisting of evergreen and deciduous trees and shrubs. North American species in the family include members of the genera *Castanea* Mill. (Chestnut), *Fagus* L. (beech), *Notholithocarpus* P.S. Manos, C.H. Cannon, & S.H. Oh (tanoak) and *Quercus* L. (oak). Several members of the Fagaceae are economically important for timber production, including species of oak, beech, and chestnut; these genera also contain many ornamental varieties. Several species in Fagaceae produce edible nuts; chestnuts are the most widely used and economically important. Species within the genus *Castanea* are listed in Table 2.1a. Further details on select species and hybrids are discussed in Section 2.1.1.

Table 2.1a. Species of the chestnut genus. *The taxonomic status of the Ozark chinquapin is disputed, with some sources placing it as a variety of *C. pumila* (*C. pumila* var. *ozarkensis*). Here, we place it at the species level following Ashe (1923) and Nixon (1997).

| Scientific Name | Common Name | Geographic Origin |
|--|----------------------|-------------------|
| <i>Castanea dentata</i> (Marsh.) Borkh. | American chestnut | North America |
| <i>C. ozarkensis</i> Ashe* | Ozark chinquapin | North America |
| <i>C. pumila</i> (L.) Mill. | Allegheny chinquapin | North America |
| <i>C. sativa</i> P. Mill. | European chestnut | Europe |
| <i>C. crenata</i> Siebold & Zucc. | Japanese chestnut | Asia |
| <i>C. henryi</i> (Skan.) Rehder. & E.H. Wilson | Henry chestnut | Asia |
| <i>C. mollissima</i> Blume | Chinese chestnut | Asia |
| <i>C. seguini</i> Dode | Seguin chestnut | Asia |
| Naturally Occurring Hybrids | | |
| <i>C. × neglecta</i> Dode [<i>dentata</i> × <i>pumila</i>] | n/a | North America |

Members of the genus *Castanea* are distributed across the northern latitudes of the world, mostly in the temperate and cooler portions of subtropical climatic zones (Figure 2.1a). Species in the chestnut genus have the potential to hybridize in zones where two or more species overlap, and

when they are planted in close proximity or intentionally crossed (Villani *et al.*, 1999; Johnson, 1988). A common factor which facilitates interspecific hybridization among chestnut species is that they are all diploids with a 2n number of 12 (Jaynes, 1962).



*Figure 2.1a. Worldwide distribution of *Castanea*, reproduced from Lang *et al.* (2006). This figure shows the historical range of species; introduced or naturalized populations are not included. This figure names multiple types of North American chinquapin as varieties of *C. pumila*; see Table 2.1a caption regarding nomenclature.*

2.1.1 North American species in the chestnut genus

Prior to the introduction of chestnut blight to North America, American chestnut was found as far north as Maine and Ontario, Canada and as far south as Georgia and Mississippi, covering an approximate 200 million acres of land (Saucier, 1973). In many areas of its natural range, especially in the southern Appalachians, American chestnut was the predominant forest tree species in terms of both stand density and stature (Buttrick, 1915; Braun, 1950).

The original range of the American chestnut overlapped the ranges of Ozark chinquapin (*Castanea ozarkensis*) and Allegheny chinquapin (*C. pumila*) (Figure 2.1a; see Table 2.1a regarding nomenclature). The chinquapins are shrubs and subcanopy trees producing one nut per bur. North American chinquapins are susceptible to chestnut blight, though reportedly less susceptible than American chestnut (Detwiler, 1915; Graves, 1950). All three species respond similarly to the disease. That is, the chestnut blight fungus forms cankers on aboveground portions of the plant; the cankers girdle and kill living stems, but the plants have the ability to sprout from the root collar and lower portion of the stem. The chinquapins occupy much of their pre-blight range, though in

reduced numbers (Johnson, 1988; USDA-NRCS, 2019). See Paillet (1993) for a further description of the growth form, life history, and response to chestnut blight of North American chinquapins.

North American *Castanea* species appear to hybridize where their distributions overlap (Figure 2.1.1a). Tucker (1975) observed an intergradation in features where Ozark chinquapins in the mountainous interior overlap with the Allegheny chinquapins on the coastal plain. In the central and southern Appalachians, numerous authors have reported plants with intermediate morphology between American chestnut and Allegheny chinquapin; this population has been described as the hybrid taxon *C. x neglecta* (Dode, 1908; Hardin and Johnson, 1985; Johnson, 1988). Other authors have described a taxon called *C. x alabamensis* (Camus, 1928; Elias, 1971), which may be another *C. dentata* x *C. pumila* hybrid, though it has also been considered an isolated population of *C. ozarkensis* (Johnson, 1988), or an entirely separate species (Ashe, 1925; Graves, 1950). More recent analyses of *C. x alabamensis* confirm it is morphologically and phylogenetically unique from *C. dentata* and not likely a hybrid, but leave its species status unresolved (Perkins, 2016; Perkins *et al.*, 2019). The evolutionary history of North American *Castanea* species is still not fully understood, and is complicated by recent and past hybridization and incomplete lineage sorting (Shaw *et al.*, 2012).

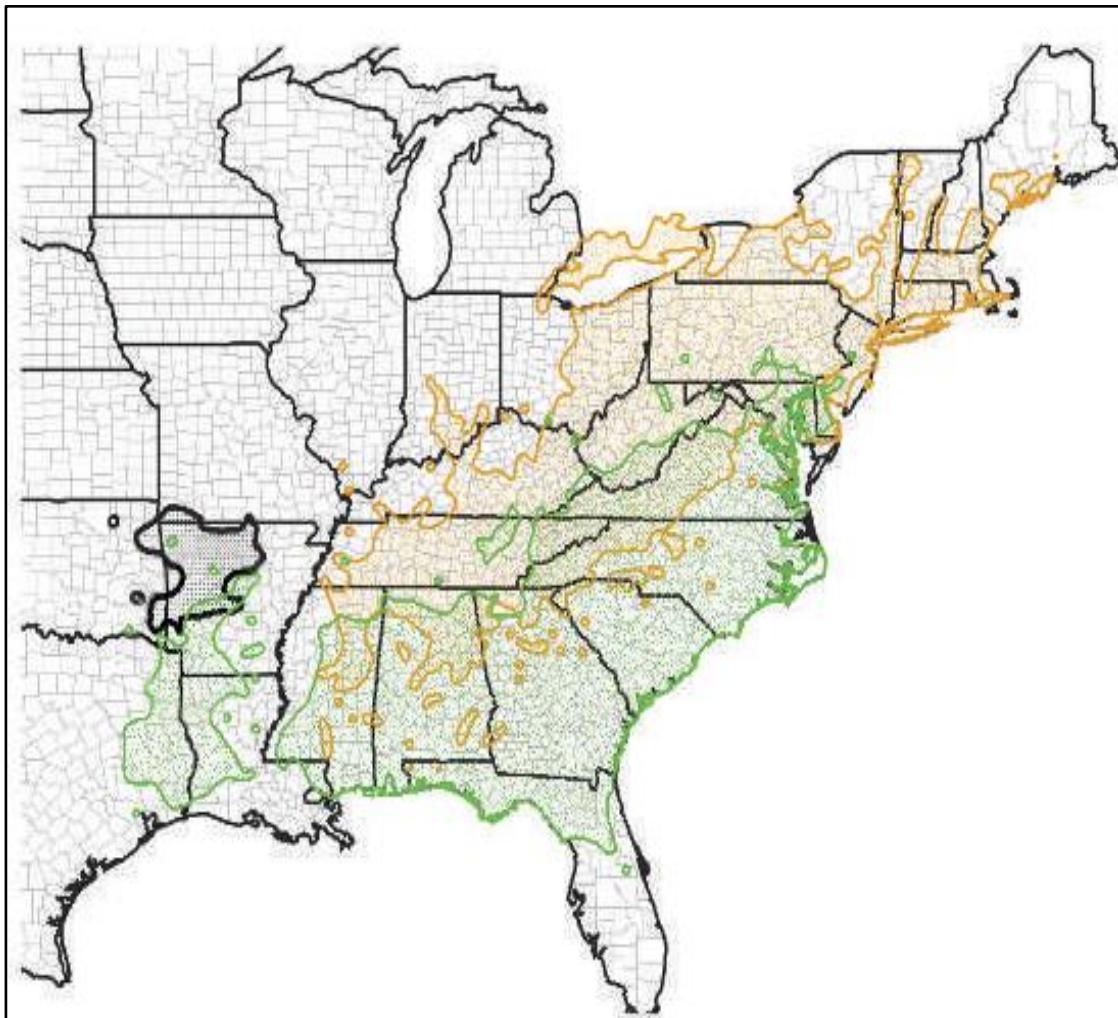


Figure 2.1.1a. Ranges of North American *Castanea* species: *C. dentata* (orange), *C. pumila* (green), and *C. ozarkensis* (black stippled). Based on Little (1977), reproduced from Shaw et al. (2012).

American chestnuts were planted outside their native range in many places in the United States for their usefulness in producing timber and wood products, nuts, and shade. Railroad companies planted thousands of chestnuts along their tracks, and chestnut was listed as a recommended tree by organizations including the Society for the Promotion of Agriculture, Arts, and Manufactures; Iowa State Horticultural Society; and the state boards of agriculture of California and Nebraska (Hough, 1878). The Civilian Conservation Corps (CCC) planted thousands of American, Asian, and hybrid chestnut trees throughout the country in the 1930s (Sandra Anagnostakis, personal communication). Some trees planted in the 1800s in the Pacific Northwest remain alive and blight-free today (Gillis, 2017). Even before European colonization, Native Americans may have assisted establishing American chestnut in northern parts of its range by transporting seeds along trade routes (Russell, 1987; Gailing and Nelson, 2017).

In the late 19th century, the range of American chestnut was contracting in the south, apparently a result of *Phytophthora* root rot, also called ink disease, caused by the oomycete *Phytophthora cinnamomi* (Russell, 1987). Environmental conditions favored trees growing in the North and in

mountainous regions that were less susceptible to *Phytophthora* root rot, compared to trees growing in the southern lowlands; the latter in large part succumbed to the disease. *P. cinnamomi* was probably introduced to the southern United States prior to 1824 (Anagnostakis, 2012). It remains a challenge to American chestnut restoration programs today (Santos *et al.*, 2017).

The range of American chestnut has expanded over the last century (Figure 2.1.1b); it is now found in the lower peninsula of Michigan (Brewer, 1995), southwestern Wisconsin (Paillet and Rutter, 1989), Illinois (Russell, 1987), Iowa (Russell, 1987; Farrar, 2001), Louisiana, Missouri, and Florida (Dalgleish *et al.*, 2016). The range of American chestnut was naturally expanding northwestward at the time of blight introduction (Brewer, 1995), though much of the observed expansion is presumably due to naturalized populations from historic plantings.

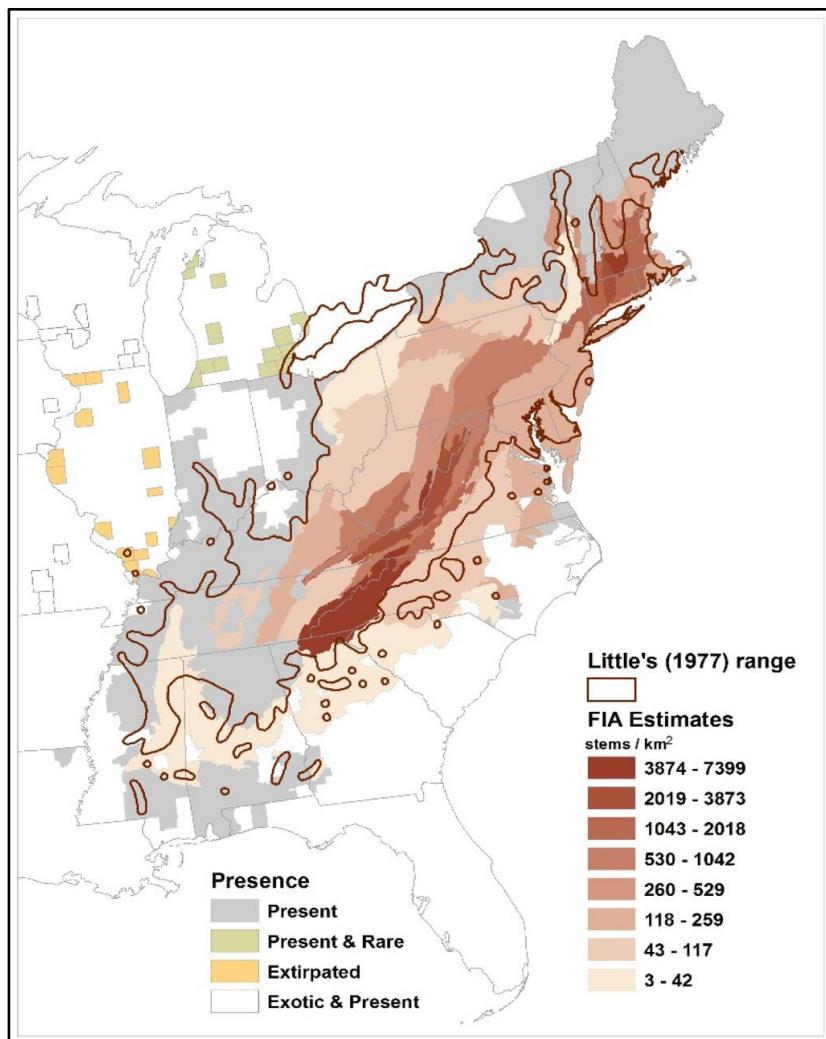


Figure 2.1.1b. Occurrence and abundance of surviving American chestnut. Outline from Little (1977), with more recent data from The Biota of North America Program (bonap.org) and the U.S. Forest Service Forest Inventory and Analysis (FIA). Reproduced from Dalgleish *et al.* (2016).

As a consequence of blight, the abundance of chestnut has drastically declined; the number of live stems today is roughly 431 ± 30.2 million, at most 10% of the pre-blight population (Dalgleish *et al.*,

al., 2016). Data on the size class distribution before the blight are limited, but it is safe to say that the current population is highly skewed toward small trees; the vast majority of stems (estimated 84%) are < 2.5cm dbh (Dalgleish *et al.*, 2016). The highest density of stems is found in the central portion of the range, including the Appalachians and southern New England. Chestnuts are found in a broad range of site conditions, including flat and sloped land (though rarely on very steep slopes); they are found on all aspects, only slightly more abundant on Northeast facing slopes; and are found mainly on mesic sites, some on xeric sites, and only rarely on very wet sites (McWilliams *et al.*, 2005). Despite the small extant population of American chestnut, a large part of the original genetic diversity has been preserved, with the highest diversity in the southern portion of the range (Dalgleish *et al.*, 2016; Gailing and Nelson, 2017). The American Chestnut Foundation (TACF) and their partners maintain hundreds of orchards, covering over 400 hectares and containing thousands of hybrid and pure American chestnut trees (Hebard, 2012; Fitzsimmons, 2017; The American Chestnut Foundation, 2019b).

2.1.2 European species, Asian species and hybrids in the chestnut genus

European, Chinese, and Japanese chestnuts have been cultivated for thousands of years for timber and nut production (Goodell, 1983; Villani *et al.*, 1994; Anagnostakis, 2012; LaBonte *et al.*, 2018). Hundreds of cultivars have been named, many of which are commercially available from nurseries (Anagnostakis, 2012). European chestnut material was imported into the United States as early as 1776 (Brooks, 1937), and Japanese chestnuts were first imported in 1876 (Anagnostakis, 2012). Chinese chestnuts were imported as early as 1901, *C. seguini* in 1917, and *C. henryi* was introduced by 1920 (Galloway, 1926). Many of these introductions were conducted by the USDA for agricultural purposes, including blight-resistant breeding stock to create Asian-American hybrids. American chestnut is sexually compatible with the European chestnut (*C. sativa*) and Henry chestnut (*C. henryi*). Interspecific crosses of American chestnut with Chinese chestnut (*C. mollissima* x *C. dentata* or the reverse) and with other Asian *Castanea* species are not universally successful (Jaynes, 1964; Section 3.3.2), but fertile offspring are produced often enough to allow hybridization programs to be constructed with the intent of mitigating chestnut blight (Burnham *et al.*, 1986; Jacobs *et al.*, 2013).

While most species of *Castanea* can freely hybridize, such crosses are not without risks: various crosses have been reported to result in problems such as abnormal nut development called Internal Kernel Breakdown (Fulbright *et al.*, 2014), a semi-lethal condition called “cracked bark” in young seedlings (Jaynes, 1964), and male sterility in hybrid offspring (Anagnostakis, 2012; Sisco *et al.*, 2014). Male sterility is apparently a result of interactions between dominant nuclear genes from Asian chestnuts and mitochondrial (or chloroplast) genes in American chestnut, and full fertility can in some cases be regained in a portion of the offspring from a male-sterile F1 hybrid (Soylu, 1992; Sisco *et al.*, 2014). We are not aware of reports of male sterility resulting from hybrid crosses among North American *Castanea* species (Section 2.1), or between these species and European chestnut (Jaynes, 1964).

European and American chestnuts were introduced to the west coast of the United States beginning in the mid-1800s. Few trees that were brought to Oregon still survive from this migration, but occasional large trees have survived in these regions in the absence of blight (Gillis, 2017). Immigrants brought European and other chestnut varieties with them during the California

gold rush. Some plantations from this era still exist in the Sierra foothills, North Coast, and Central Valley (Vossen, 2000) but there are no reports of chestnuts becoming naturalized in these areas.

There is a nascent chestnut industry in the United States made up of mostly hybrid chestnuts (mixes of *C. mollissima*, *C. crenata*, *C. sativa*, and *C. dentata*). Asian chestnut species and hybrids provide better blight tolerance and produce larger nuts than American chestnuts; larger nuts are more marketable in the United States (Facciola, 1998; Olsen, 2000; Gold *et al.*, 2005; Hochmuth *et al.*, 2018). Some pure American chestnuts are grown commercially, meeting the demand for native species. The U.S. produces < 1% of the world's chestnut crop (Vossen, 2000) from approximately 900 farms covering approximately 2,200 acres at bearing age (USDA-NASS, 2017). Most chestnut farms are in Michigan, California, Florida, and Pennsylvania, with only Pennsylvania in the natural range of the American chestnut (Agricultural Marketing Resource Center, 2018). There are also likely many small-scale "hobby growers" of chestnut, as Chinese, hybrid, and pure American seedlings have been available from various sources and may be freely shared between growers.

With one notable exception in Windham County, Connecticut, we know of no reports in the scientific literature of naturalized Asian or hybrid chestnut populations in North America. In Connecticut, a population 72 Chinese chestnut trees was found within a second growth mixed forest which had regenerated naturally from an abandoned agricultural field (Jaynes, 1967; Miller *et al.*, 2014). The forest was adjacent to an orchard containing Chinese chestnut trees originally planted in 1926; all of the Chinese chestnuts in the forest were offspring of the nearby orchard trees. Miller *et al.* (2014) concluded that the establishment of the Chinese chestnuts on this site was facilitated by shallow soil that limited the height of competing trees, combined with a rare window of low seed and seedling predation. All the trees were around 50 years old at the time of the study, comprising a single cohort. No 3rd generation seedlings have become established, presumably due to unfavorable site conditions including increased herbivory and competition.

2.2 Biology and silvics of American chestnut

The American chestnut is a long-lived, fast-growing, late-successional hardwood tree. It is a generalist, adapted to a broad range of environmental and climatic conditions (Jacobs *et al.*, 2013; Wang *et al.*, 2013). Where it was most abundant, chestnut often grew in association with oak, hickory, basswood, tuliptree, and beech, with an ericaceous shrub understory (Braun, 1950).

American chestnut trees grow preferentially on well-drained, sandy, and slightly acidic (i.e. pH of 5 to 6) soils, often on slopes and ridges (Braun, 1950; Russell, 1987). Flat areas, alkaline or limestone-derived soils, and very wet or dry soils, are not conducive to chestnut colonization (Paillet, 2002).

American chestnut is classified as an intermediate shade-tolerant to shade-intolerant species (Toumey and Korstian, 1947; Joesting *et al.*, 2009; Jacobs *et al.*, 2013). Established seedlings and stump sprouts can persist for years in low light conditions (Paillet and Rutter, 1989; McEwan *et al.*, 2006), but grow quickly when released into high light conditions, with high photosynthesis rates matching or exceeding many shade intolerant, fast growing species (Joesting *et al.*, 2009).

Because we have no contemporary studies of healthy populations of American chestnuts within their native range, and must rely largely on historical records, knowledge of basic biological

characteristics and ecological interactions are limited, especially regarding mature trees. Buttrick (1925) reports that American chestnut lived several hundred years, at least in the southern Appalachians; Zon (1904) reports that chestnut trees live to an age of 400 to 600 years, though trees over 100 years grow hollow in the center. American chestnut trees were commonly recorded at heights of 70 to 100 feet, with diameters of 3 to 5 feet or more (Detwiler, 1915; Buttrick, 1925; Smith, 2000; Figure 2.2a).

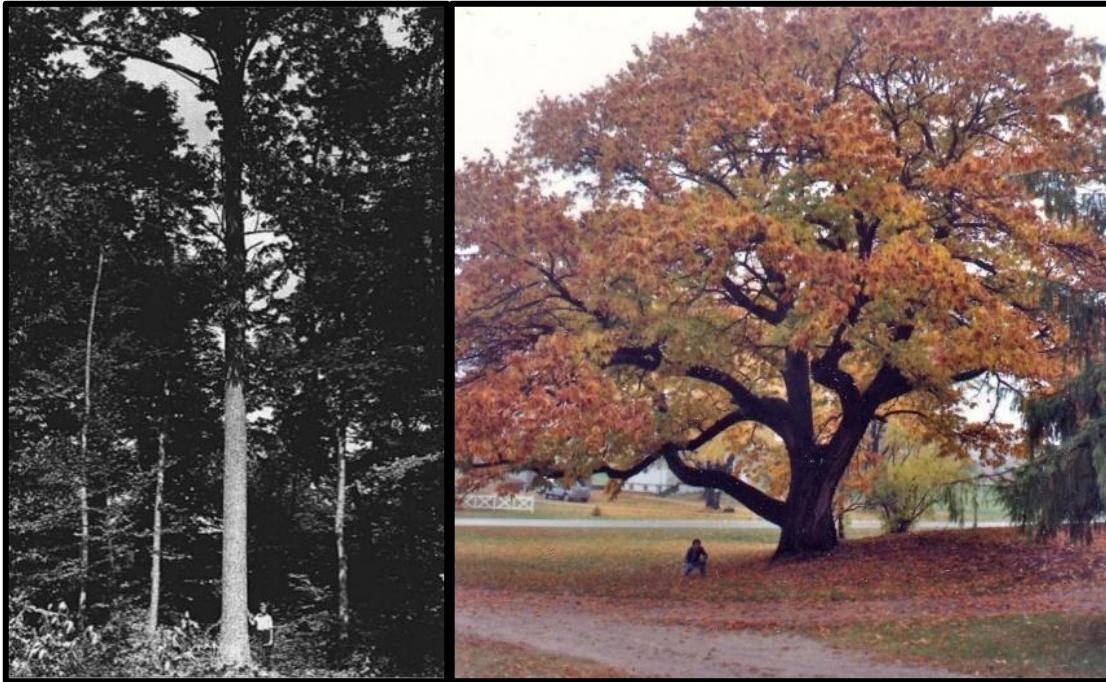


Figure 2.2a. Photos of American chestnut. At left, timber-type American chestnut photographed in 1905 near Scotland, CT, recorded as 83' tall, 27" diameter, and 103 years old (reproduced from Anagnostakis, 2012). (See center photo on "Certification" page for another example of a historically large American chestnut tree.²) At right, spreading American chestnut tree in Michigan (courtesy of Alan Hart).

2.2.1 American chestnut physical characteristics

Saucier (1973) has provided a useful description of American chestnut morphology, quoted here. See Figure 2.2.1a for photographic examples.

"The leaves of American chestnut are lance shaped and coarsely toothed, with the bristle-tipped teeth pointing forward sharply. They are about 2 inches wide and 5 to 8 inches long. The petiole is short and stout and is enlarged at the base. Flowers are borne on ascending spike-like aments that are either staminate or bisexual. The staminate aments are about 5 inches long, and the flowers are in clusters of three to seven along the ament axis. Pistillate flowers are in clusters of two or three at the base of shorter bi-sexual aments. The fruit is an edible nut $\frac{1}{2}$ to 1 inch in diameter. It is nearly flat on one or both sides, and is borne in clusters of two or three in a bur covered with sharp, branched spines. Twigs are slender to moderately stout. They

² Center photo on "Certification" page courtesy of Hurley Heritage Society, Hurley, NY.

are normally smooth, chestnut brown, and somewhat lustrous. The pith is star shaped. Lateral buds are about $\frac{1}{4}$ inch long, ovoid, brown, and have two or three visible scales. There are no terminal buds. The bark is dark brown and shallowly fissured into broad, flat ridges.”

In the now endemic presence of the chestnut blight fungus, the American chestnut growth form has been reduced from a dominant overstory tree to a small understory shrub (Elliott and Swank, 2008; Dalgleish *et al.*, 2015). Sprouts will emerge from the root collar of diseased trees, but these shoots will themselves succumb to blight. While young shoots less than 50 cm in height are only rarely infected, disease incidence increases steadily with stem size (Davelos and Jarosz, 2004). Generally, chestnut stems will die of blight infection before they reach 15 m of height or a diameter of 20 cm, sooner in areas where blight spores are numerous, such as places with a high density of chestnut sprouts (Paillet, 2002). While individual chestnut clones may survive for many years through continual resprouting, blight infection increases the likelihood of the death of the entire clone, especially when combined with competition and/or abiotic stress such as drought (Griffin *et al.*, 1991; Parker *et al.*, 1993).



Figure 2.2.1a. Physical characteristics of American chestnut. a. Closed and partially open burs. b. Seeds within bur, generally three per bur. c. Mature leaves. d. Mature bark. e. Mature flowers (catkins).

2.2.2 Natural reproduction and dispersal mechanisms of American chestnut

American chestnuts are monoecious (bearing both male and female flowers on the same plant). Depending on location, flowering usually occurs in June to early July (Paillet, 2002; Horton, 2010). From our observations in central New York, pollination usually occurs at the end of June or the first week of July. Individual female flowers are receptive for about 2 – 3 days and a whole tree may have receptive female flowers for up to two weeks.

American chestnuts are primarily wind pollinated (Clapper, 1954; Johnson, 1988). Though not essential for pollination, insects, especially bees, likely play a role (Clapper, 1954), and multiple bee species have been observed visiting catkins on American chestnuts and other chestnut species (de Oliveira *et al.*, 2001; Giovanetti and Aronne, 2011; Hasegawa *et al.*, 2015; Tumminello, 2016;

Zirkle, 2017). Exclusion of insects was shown to reduce seed production in both European chestnut (de Oliveira *et al.*, 2001) and Ozark chinquapin (Zirkle, 2017).

Chestnut trees need to be within 30 to 100 m apart to have high pollination rates, and trees further than 300 to 400 m apart will generally not pollinate each other (Forest *et al.*, 1977; Cook and Forest, 1979; Russell, 1987; Rutter, 1990). Chestnut pollen can travel up to 100 km (Fernandez-Lopez and Alia, 2003), but due to rapid desiccation, pollen viability decreases with time spent in the air, so effective pollination distances are much shorter.

Chestnut species are considered self-incompatible (Clapper, 1954; Russell, 1987) although there is at least one report that self-fertilization may occur rarely in American chestnut, with < 1% to perhaps < 5% of the tree's flowers (Rutter, 1990). One study of Japanese chestnut (*C. crenata*) reported 0.3% of nuts had been self-pollinated (Hasegawa *et al.*, 2009). At least one strongly self-fertile hybrid has been reported (Anagnostakis, 2014). American chestnut can outcross to other chestnut species, including Chinese chestnut (*C. mollissima*), Japanese chestnut (*C. crenata*), European chestnut (*C. sativa*), and chinquapin (*C. pumila*) (Jaynes, 1964) to form hybrids (Section 2.1.2).

The time to seed production may vary depending on conditions such as light availability and soil type. Zon (1904) reported that chestnuts in Southern Maryland produced seed at 8 – 10 years, but "regular and plentiful crops" would be produced only after the 20th year. Similar timeframes were reported for trees in New York by Cook and Forest (1979). Naturalized trees growing under a forest canopy in Wisconsin produced seed after about 20 years (Paillet and Rutter, 1989). Post-coppice shoots have been reported to produce seed sooner than seedlings (Zon, 1904; Wang *et al.*, 2013), though we have not observed any substantial difference in flowering time on coppiced shoots in our central New York orchards. Light availability accelerates growth and hastens flowering; optimal conditions can shorten the age of seed production to as little as 4 (Wang *et al.*, 2013) to 6 (Zon, 1904) years.

Seeds typically mature in late September or October. Fall frost serves to open the burs and release the seeds, which must go through natural or artificial cold stratification before sprouting the following spring (Bonner, 2008; Wang *et al.*, 2013). Chestnut seeds do not survive multiple years in natural conditions, so there is no seed bank (Davelos and Jarosz, 2004). Due to their high desirability to insects and wildlife, actual germination and establishment of chestnut seedlings may be a rare occurrence (Detwiler, 1915; Hawley and Hawes, 1918; Toumey and Korstian, 1947).

Chestnut species rely on animals for seed dispersal beyond the immediate vicinity of the parent tree. Rodents, including squirrels (*Sciurus* spp.), eastern chipmunks (*Tamias striatus*) and mice (*Peromyscus* spp.), cache seeds for future consumption, including American chestnut (Toumey and Korstian, 1947; Lichti *et al.*, 2014). Unrecovered seeds may germinate and become established seedlings. Other species of birds and mammals likely also play a role, including blue jays (*Cyanocitta cristata*) (Darley-Hill and Johnson, 1981; Johnson and Webb, 1989), crows (*Corvus brachyrhynchos*) (Zon, 1904), and, historically, the now-extinct passenger pigeon (*Ectopistes migratorius*) (Webb, 1986). Whole burs are possibly transported by large mammals such as black bear (*Ursus americanus*) and white-tailed deer (*Odocoileus virginianus*) by hitchhiking on the animal's fur (Wang *et al.*, 2013).

Chestnut seedlings that are able to germinate are subject to herbivory and damage by native mammals including white tailed deer, various rodents, and cottontail rabbit (*Sylvilagus florianus*), native insects including chestnut sawfly (*Craesus castaneae*) and periodical cicadas (*Magicicada* spp.), as well as non-native insects including Asiatic oak weevil (*Cyrtepistomus castaneus*) and chestnut gall wasp (*Dryocosmus kuriphilus*) (Cook and Forest, 1979; Clark *et al.*, 2014; 2016).

American chestnut does have the ability to regenerate vegetatively by sprouting new shoots from the root collar; this allows individual chestnut genets to remain alive even in the presence of blight, and allows regeneration after fire (Hawley and Hawes, 1918; Toumey and Korstian, 1947; Paillet, 2002) or logging (Buttrick, 1915; Faison and Foster 2014). The ability of chestnut trees to generate sprouts declines with stem age, but trees over 100 years old may retain the ability to produce sprouts (Zon, 1904; Russell, 1987). Chestnut does not sprout from roots (Paillet, 1984, 1993), so new shoots will be in the immediate location of the former tree; natural dispersal to a new location can take place only by seed.

2.2.3 American chestnut and fire

The literature on the fire ecology of American chestnut is somewhat conflicting. Many early authors, referenced by Paillet (2002) and Wang *et al.* (2013), cited chestnut's thin bark and shallow root system as indications of fire susceptibility, suggesting that frequent fire limits or prevents chestnut recruitment. Chestnuts allocate more biomass above-ground (> 70%) than below-ground; that ratio is similar to other typically fire-susceptible species (Wang *et al.*, 2006). In contrast, Zon (1904) describes chestnut as a "deep-rooted species," which allows it to withstand surface fire. Additionally, American chestnut leaf litter is especially flammable, which can facilitate relatively low-intensity surface fires typical of other pyrophytic tree species (Kane *et al.*, 2018). If chestnut clones are able to survive a fire, vigorous sprouting and rapid response to light allow them to capitalize on the reduced competition. Sediment core analysis suggests that historic and prehistoric fires served to favor chestnut and oak over competing tree species (Delcourt and Delcourt, 1998; Foster *et al.*, 2002).

Paillet (1984) found that an intense ground fire that occurred before the growing season on a Massachusetts site killed all the above-ground chestnut stems. However, the fire did not kill the entire organism, which responded by sprouting from the root crown. In Virginia, regrowth from established chestnut trees was not negatively affected by fire, and the increased light availability enhanced growth (Vaughan, 2018).

McCament and McCarthy (2005) found that chestnut seedlings planted in a mixed-oak forest did best on sites that were thinned and burned before planting, as compared to thinned only, burned only, or no treatment. In that study, the seedlings were not subjected to fire themselves, but another study by Belair *et al.* (2014) exposed chestnut seedlings to fire and found that they showed consistent sprouting ability similar to that of northern red oak (*Quercus rubra*).

Kane *et al.* (2018) tested relative flammability of dried leaves from American chestnut, Chinese chestnut, and backcross hybrids. They found that Chinese chestnut litter was less flammable than that of American chestnut, and backcross litter was generally intermediate between the two. This

has implications for forest management in areas that were formerly dominated by American chestnut, as less flammable litter could ultimately lead to scarcer but more intense fires.

Overall, the evidence suggests that fire has historically enhanced American chestnut's ability to compete against less fire-adapted tree species (Foster and Zebryk, 1993), but the response of chestnut to fire is likely complex and dependent on fire intensity and timing (Wang *et al.*, 2013), and further research is warranted.

2.3 Ecological consequences of the loss of American chestnut

In areas where it predominated, American chestnut was considered a foundation species, having a profound effect on population and community dynamics and modulating ecosystem processes (Paillet, 2002; Ellison *et al.*, 2005). The structure and diversity of Eastern forests have been altered through the replacement of American chestnut with other species, altering forest productivity, nutrient cycling, and community dynamics in both terrestrial and aquatic environments (Ellison *et al.*, 2005; Elliott and Swank, 2008; Dagleish and Swihart, 2012).

2.3.1 Replacement of American chestnut by other tree species

Chestnuts were replaced mainly by oak and maple, and to a lesser extent, hickory, birch, black cherry, tulip tree, and others as dominant canopy tree species (Braun, 1950; Keever, 1953; Stephenson, 1986; Stephenson *et al.*, 1991; Brewer, 1995). No single species has emerged in a dominant role across a broad geographic range, comparable to the pre-blight status of American chestnut.

In the Ceweeta Basin, Southern Appalachian Mountains of North Carolina, where chestnut had been clearly dominant, chestnut oak (*Quercus montana* and/or *Q. michauxii*)³ and red maple (*Acer rubrum*) became the most important species and were found in all environmental conditions. Other species replaced American chestnut in specific habitats, such as Eastern hemlock (*Tsuga canadensis*) near streams at low- to high-elevations, and tulip tree (*Liriodendron tulipifera*) in moist coves. Flowering dogwood (*Cornus florida*) and sourwood (*Oxydendrum arboreum*) also increased in importance, collectively resulting in increased plant diversity in this area after chestnuts declined (Elliott and Swank, 2008).

On Salt Pond Mountain in southwestern Virginia, northern red oak, which was only 11% of the canopy in 1932, has taken over as the most dominant species. Additionally, several species were found in the canopy that were not recorded as canopy trees in 1932: sugar maple (*Acer saccharum*), serviceberry (*Amelanchier arborea*), sweet birch (*Betula lenta*), black cherry (*Prunus serotina*) and black locust (*Robinia pseudoacacia*) (Stephenson, 1986).

In the Blue Ridge mountains in North Carolina, Keever (1953) found northern red oak, chestnut oak, hickory (*Carya ovalis* and/or *C. glabra*), and white oak (*Quercus alba*) to be the most dominant trees, and suggested that the oak-chestnut forest type (*sensu* Braun, 1950) would be replaced by

³ Papers cited in this section (Elliott and Swank, 2008; Keever, 1953; Stephenson, 1986; Stephenson *et al.*, 1991; Woods and Shanks, 1959) use the scientific name *Quercus prinus*. Contemporary authors regard the name *Q. prinus* of uncertain position, referring to either *Q. montana* (chestnut oak) or *Q. michauxii* (swamp chestnut oak). Discussions of "Q. prinus," "Q. montana," and "chestnut oak" in the context of chestnut ranges are likely referring to the same species, as *Q. michauxii* is not found in mountainous settings in the southern Appalachians.

an oak-hickory forest. This hypothesis was borne out on at least one location: Beanfield Mountain, Virginia, where an oak-hickory association emerged roughly half a century after the demise of American chestnut (McCormick and Platt, 1980). However, a study contemporary to Keever's in the Great Smoky Mountains of eastern Tennessee and western North Carolina found that chestnut oak, northern red oak, and red maple were the most common trees to replace chestnut, with hickory comprising only 1% of the replacements (Woods and Shanks, 1959). Several studies summarized by Stephenson (1986) suggest that hickory increased only in some localities, and is not of major importance throughout the southern Appalachians. Overall, Stephenson found that chestnut oak and northern red oak were the most abundant trees to replace American chestnut in the southern Appalachians, though no single tree species has achieved chestnut's former dominant or codominant role throughout the region.

Patterns of replacement were somewhat different in the northern portion of chestnut's range. On a former Oak-Chestnut forest in Pennsylvania, the dominant tree 50 years after the elimination of chestnut was black cherry, followed by red maple, sugar maple, black oak (*Quercus velutina*), sweet birch, and sour gum (*Nyssa sylvatica*) (Mackey and Sivec, 1973). Salvage logging of chestnut and some other species created an open environment favoring early- and mid-successional species, and the authors predict that the forest will continue to change, with black cherry and red maple being replaced by more shade-tolerant species.

Ireland *et al.* (2011) found that birch (*Betula* spp.) was the most significant tree to replace chestnuts on a site in Massachusetts. This pattern agrees with observations in New Jersey, where an increase of black birch was the only change tied directly to the elimination of chestnut (Good, 1968). Brugam (1978) also found a pattern of birch replacement of chestnut on a site in Connecticut, but birch was replaced by oak after a period of about 20 years. Bradshaw and Miller (1988) saw an increase in the dominance of Eastern hemlock, black birch, red maple, northern red oak, and white oak in a Massachusetts following the decline of American chestnut.

Wherever American chestnut was an important canopy tree before the blight, chestnuts were still found. They persisted as stumps and small trees, often multi-stemmed, as a result of the disease, and did not occupy a dominant canopy position (Keever, 1953; Woods and Shanks, 1959; Good, 1968; Mackey and Sivec, 1973; Stephenson, 1986; Elliott and Swank, 2008; Ireland *et al.*, 2011).

2.3.2 American chestnut as a food source

Pre-blight American chestnut has been described as "the most important wildlife plant in the eastern United States" (Davis, 2005). American chestnut produced a heavy mast crop of calorie-packed seeds. Chestnut mast production in pre blight forests was not directly measured, but estimates range from 270 kg/ha to 2500 kg/ha (Diamond *et al.*, 2000; Gilland *et al.*, 2012). American chestnut disappeared from forests before many systematic studies of wildlife food habits were undertaken (Hill, 1994), but we know that game animals such as wild turkeys (*Meleagris gallopavo*), white-tailed deer, and black bears all consumed the annual chestnut crop (Diamond *et al.*, 2000; Wang *et al.*, 2013), as well as many other vertebrates including rodents (Lichti *et al.*, 2014) and birds, such as the extinct passenger pigeon (Webb, 1986) and heath hen (Hill, 1994).

Unlike most North American hard mast species, whose production varies from year to year, chestnut is relatively stable and a mast failure (a year with no nut production) is probably extremely rare for chestnut within its native range (Diamond *et al.*, 2000). Post-blight forests in the range of the American chestnut, therefore, produce hard mast at levels that are lower overall, and more highly variable than they were in pre-blight forests. Dalgleish and Swihart (2012) used available data on basal area and mast production for 7 hardwood trees to model the changes in mast production and population of consumer species in pre- and post-blight forests. They found that as American chestnut populations declined in dominance due to chestnut blight, oak species generally took their place as the leading mast-producing trees. When this happened, estimated total mast production in southern (North Carolina) and northern (Connecticut) sites decreased 35% and 80%, respectively, and variation increased 60% and 76%, respectively. In the model, populations of white-footed mice (*Peromyscus leucopus*), chipmunks, squirrels, and deer all declined. White-footed mice populations declined the most (nearly half in years of low alternative food sources), while deer populations showed the smallest change.

American chestnut provided a food source to numerous insect species, especially during the flowering period. Pollen feeders in the insect orders coleoptera (beetles), lepidoptera (moths) and hymenoptera (bees) have been observed visiting chestnut catkins (Clapper, 1954; de Oliveira *et al.*, 2001; Giovanetti and Aronne, 2011; Opler, 1978; Hasegawa *et al.*, 2015; Tumminello, 2016; Zirkle, 2017). In addition to these observed interactions, chestnut pollen has been shown to be especially nutritious to bumble bees (*Bombus terrestris*) (Tasei and Aupinel, 2008). There is evidence suggesting some native insect species followed the tree to functional extinction (Opler, 1978).

A study by Smock and MacGregor (1988) on nutritional quality of leaf litter showed that American chestnut leaf litter is of similar quality to that of pignut hickory (*Carya glabra*), both of which are of better nutritional quality than northern red oak leaf litter. In a laboratory experiment, leaf shredding herbivorous stream invertebrates preferentially grazed on chestnut and hickory leaf litter as compared to oak, and growth rates were faster on chestnut and hickory than on oak. Oaks are the most important tree species to replace chestnut, with hickory and other species locally important in some areas (Smock and MacGregor 1988; Section 2.3.1). In areas where oaks replaced chestnut, the change in leaf litter quality would have directly affected leaf shredding insect populations, as well as downstream effects such as reduction in fine particulate organic matter released as a byproduct of feeding (Smock and MacGregor 1988).

Preliminary laboratory tests of aquatic insect herbivory on various types of deciduous leaves indicated that mayfly (*Frenesia difficilis*) larvae preferred American chestnut leaves to most other leaves commonly found in extant forests. Nine leaf types were studied: American beech (*Fagus grandifolia*), American chestnut, American sycamore (*Platanus occidentalis*), chestnut oak (*Quercus montana*), combined red and sugar maple, shagbark hickory (*Carya ovata*), tulip tree, and white oak. Only maple and shagbark hickory were preferred over American chestnut by mayfly larvae (B. Sweeney, J. Jackson, and D. Funk, unpublished data).

The changes in food availability resulting from the loss of American chestnut had far reaching effects, and may have contributed to more unstable, less resilient community dynamics (Kelly *et al.*, 2008; Dalgleish and Swihart, 2012).

2.4 Introduction, introgression, and invasive potential of American chestnut

The speed and extent to which a blight-tolerant American chestnut would become established as a dominant canopy tree in today's eastern US forests are difficult to predict. Experimental data simply do not exist due to the lack of healthy American chestnuts in natural habitats over the past century. In the decades since the introduction of chestnut blight and the loss of American chestnut as a canopy tree, eastern forests have reached a new ecological state, and any discussion of the introduction of a blight-tolerant American chestnut, whether by selection, hybrid breeding, mutagenesis, or genetic engineering, should include an evaluation of its potential to invade and disrupt the eastern forest as it exists today. This subsection uses historical data and current research to explore implications and likely rates of introgression of a blight-tolerant American chestnut if it were reintroduced in the near future.

Since much of the northeastern US was covered by glaciers about 10,000 to 20,000 years ago, and much of that area was subsequently colonized by tree species that immigrated from southern refugia, we can infer immigration rates from post-glacial data. The rate of range expansion after the last ice age has been estimated for several tree species using fossil pollen data: American chestnut spread at an average rate of 100 m/yr., which was the slowest of all species studied. By comparison, oaks spread at an average rate of 350 m/yr., and American beech spread at an average rate of 200 m/yr. (Davis, 1981; Davis, 1983). A separate pollen analysis in New England reveals that *Castanea* appeared in the region much more recently (~2000 years ago) than other deciduous trees like maple and oak (> 9000 years ago), suggesting chestnuts were relatively slow to spread north after glaciers receded (Foster and Zebryk, 1993). The authors note that *Castanea* tended to become dominant after it was introduced, but this may have been influenced by human activity such as clearing and burning, which began around the same time period. Multiple molecular analyses have generally confirmed these pollen studies, concluding that *Castanea* likely spread slowly northward from glacial refugia in the southeastern US (Li and Dane, 2013, Gailing and Nelson, 2017).

There is one well-studied example of American chestnut becoming naturalized outside its native range. This may give some clues as to how a blight-tolerant variety could become established following its reintroduction to its native range. A privately owned woodland near West Salem, WI was planted with a few American chestnuts from seed around 1880, before the arrival of blight to America (Joesting *et al.*, 2009; McEwan *et al.*, 2006; Paillet and Rutter, 1989). The stand is separated from the native range by several hundred kilometers, and remained free of blight well into the 20th century. Seeds from the original plantation trees germinated in the nearby forest, and established a population of approximately 5000 trees > 2.5 cm DBH (Joesting *et al.*, 2009).

On the West Salem site, chestnuts spread into the forest through a pattern of "pioneer" trees germinating 100m or more from the nearest seed source, followed by numerous saplings becoming established nearby (Paillet and Rutter, 1989). Recruitment was sporadic, with a pulse of seedlings becoming established beginning in the 1970s. This was apparently facilitated by the removal of cattle, which preferentially graze American chestnut and prevent seedling establishment, as well as logging, which freed seedlings from light competition (McEwan *et al.*, 2006). American chestnut grew considerably faster than other hardwoods on the same site (McEwan *et al.*, 2006). Data from the West Salem stand were used in a modeling study that

estimated individual American chestnut trees would produce less than one viable offspring per year until they were greater than 17 years old, and even trees > 70 years old would produce less than 5 offspring per year (Rogstad and Pelikan, 2014; Section 11.6).

In the 70 years since the original trees on the West Salem site began bearing seed, chestnut trees spread at an average rate of "no more than a few kilometers per century," (Paillet and Rutter, 1989) though the rate of spread appeared to be increasing with increased seed production by the established trees. This study indicates that American chestnut has the ecological capacity to achieve canopy dominance on favorable sites, but that it may take a century or more for blight-tolerant chestnut trees to become dominant after the first pioneer trees become established in a given area. The site's soil, topography, and climatic conditions are similar to areas where chestnut was dominant within its native range; nonetheless, this study consisted of a single site outside the native range, so generalizations should be made cautiously. This natural experiment on the naturalization of healthy American chestnut in a mixed oak woodland was cut short by the introduction of blight around 1987.

The ultimate status of American chestnut, which may take hundreds of years to realize, will depend on future land management decisions, as well as biotic and abiotic interactions that were not present before blight introduction. Disturbance regimes will affect American chestnut's ultimate role in the forest. There is uncertainty regarding the ecological characteristics of American chestnut, but there is evidence that it requires regular disturbance (such as fire, cutting, or windthrow) to maintain high abundance levels (Foster *et al.*, 2002). The use of fire by Native Americans significantly altered the Pre-columbian landscape, increasing the importance of fire adapted species such as chestnut and oak (Delcourt and Delcourt, 1997). Fires continued to shape the landscape into the 18th and 19th centuries, combined with widespread clearcutting. Chestnut is better adapted to intensive logging than its competitors due to its ability to sprout vigorously from cut stumps, so the heavy commercial logging of the 19th century served to increase the dominance of chestnut, especially in the northern portion of its range (Faison and Foster, 2014).

The current regime of fire suppression, beginning around the 1920s, has shifted Eastern forests toward more mesic conditions with increased tree density, favoring shade-tolerant, fire susceptible species such as maple, cherry, birch, and hemlock over fire adapted species such as chestnut and oak (Abrams, 2003; Nowacki and Abrams, 2008). Use of controlled fires has been suggested as a way to promote the establishment of chestnut (Jacobs *et al.*, 2013; Vaughan, 2018). Use of prescribed fire is gaining acceptance as a management tool (Arthur *et al.*, 2012), but its use may be limited by public resistance related to air quality and risk of fire escape (Jacobs *et al.*, 2013; D'Amato *et al.*, 2017).

Chestnuts are relatively shade tolerant and can persist for many years in a closed canopy until released by fire, logging, natural canopy mortality, windthrow, or other processes that increase light availability. However, chestnut may never achieve a status comparable to its pre-blight abundance without high-severity disturbance such as that provided by regular fires and/or intensive logging.

Recruitment of chestnut seedlings will also be affected by herbivory, especially by white tailed deer (Clark *et al.*, 2016). Deer populations are higher today than in the 19th and 20th centuries, and likely higher than in Pre-columbian times (Russell *et al.*, 2001), which may hinder survival of

chestnut seedlings. However, the effect of deer may be complex and context-dependent, as deer may also increase chestnut success by limiting competition (Dagleish *et al.*, 2015).

The natural spread of blight-resistant chestnut in modern forests was modelled by Gustafson *et al.* (2017). They found that the speed of spread or introgression will largely be dependent on the extent and effectiveness of restoration planting efforts. That is, without human assistance, blight-resistant trees will not spread quickly. In a scenario involving a 100-year program of aggressive chestnut reintroduction planting, chestnut could achieve a dominant position in the landscape similar to its status before the blight. However, without active restoration efforts, the rate of biomass increase would be very slow, and chestnut would take over a millennium to fully occupy the landscape. This prediction is in accordance with the general pattern observed by Richardson (1998) that the rate of invasion of exotic trees is usually correlated with the extent and duration of planting.

Blight-tolerant American chestnut is unlikely to display the highly competitive and fast spreading behavior associated with invasive species. In a model created by Reichard and Hamilton (1997), the single most reliable predictor of whether a species would be invasive in North America was whether the plant was known to be invasive elsewhere in the world. Chestnuts have been introduced throughout the United States, as well as in Argentina, Australia, and New Zealand (Jaynes and DePalma, 1984). American chestnut has become naturalized in only a few places outside its native range (Section 2.1). There are no known reports where people consider them aggressive or weedy, nor does it appear on any list of invasive plants or weeds. American chestnuts have been planted on the west coast of the United States for well over a century, and have not established any naturalized populations (Section 2.1.1). Though climatic differences between the east and west coasts may be a partial explanation, the Pacific Northwest's climate is sufficiently suitable to produce apparently healthy trees over 150 years old (Gillis, 2017).

In summary, American chestnut is a generalist species with competitive growth rate, intermediate shade tolerance, and ability to regenerate vegetatively from the root collar. These traits should allow blight-tolerant American chestnuts to become established and colonize new areas where soil and climatic conditions are suitable, which includes much of the eastern United States and southeastern Canada. However, given the historically slow spread rates, low propagule pressure, and need for disturbance to provide sufficient light for fast growth, the rate of increase would be very slow without human assistance, requiring centuries before chestnut becomes a significant presence in the landscape. Additionally, due to land use changes, American chestnut may ultimately achieve a level of abundance much lower than it had prior to blight introduction. Given these considerations, blight-tolerant American chestnut is not expected to exhibit weedy characteristics or constitute a plant pest risk.

2.5 Ecological consequences of American chestnut reintroduction

The composition of pre-blight forests may serve as a starting point for estimating the impact of introducing blight-tolerant chestnuts. However, the forests of today differ greatly from the forests of a hundred years ago in land use, species composition (including many nonnative species not formerly present), fire regimes, and climate change. Thus, predictions must be granted a high degree of uncertainty. Research by Barnes and Delborne (2019) specifically examines predicted habitat suitability and restoration implications for American chestnut based on climate models.

They conclude that climatically suitable American chestnut habitat will essentially shift northward over the next ~60 years, and recommend that restoration plans should take this into account. As with the remainder of this subsection, these predictions and consequences are generally applicable to any American chestnut restoration effort regardless of the means of achieving blight tolerance; they are not related specifically to transgenes.

With an increase in American chestnut, the importance of some co-occurring tree species would gradually but necessarily decline, as they must have as American chestnut expanded from glacial refugia in southeastern North America into its current range over the last few thousand years (Section 2.4). The most impacted species would likely be the same trees that previously coexisted with, and then replaced (after chestnut blight introduction), American chestnut. Chestnut will likely replace other tree species in proportion to their abundance, rather than replacing a single species or genus (Gustafson *et al.*, 2017). We have no reason to believe that the reintroduction of American chestnut would result in reduction of any competing tree species to threatened or endangered levels.

If blight-tolerant American chestnut were to become established as an important canopy tree, it would begin to influence ecosystem structure and function in these areas, as it did prior to the blight (Paillet, 2002). An increase in stable hard mast production would likely result from the reintroduction of American chestnut, which in turn could result in population increases of species that feed on chestnut, including small mammals, deer, black bear, wild turkeys, and several other bird species (Hill, 1994). This increase would be most pronounced during years of low seed production by other masting species (such as oaks), resulting in less fluctuation in consumer species (Dagleish and Swihart, 2012). Higher rodent populations could increase the pest potential of these species, and human-wildlife interactions could increase with greater large mammal populations.

Other indirect and complex consequences will result from changes in consumer populations. Potential effects of higher rodent populations could include increased pressure on songbirds as generalist predator populations increase, reduced gypsy moth (*Lymantria dispar*) outbreaks (moth pupae are eaten by mice), and increased Lyme disease risk to humans (Dagleish and Swihart, 2012).

Chestnut is fast growing, and its wood is slow to decay due to high tannin concentrations (Wang *et al.*, 2013). These features may provide rapid sequestration of carbon and nutrients, increasing long term carbon storage, especially when used in afforestation (Ellison *et al.*, 2005), though the magnitude of this effect may be minor (Gustafson *et al.*, 2017).

Chestnut leaf litter may alter terrestrial and aquatic ecology. There is some evidence that chestnut leaf litter has allelopathic effects, suppressing competitors including eastern hemlock and rhododendron (Vandermast *et al.*, 2002), but allelopathy was not observed in a recent study on germination of other native seeds (Section 9.1.2). Chestnut leaf litter decomposes more rapidly in the first year than oak or cherry leaf litter, and soils with chestnut leaf litter were shown to have lower N leaching rates and greater dissolved organic carbon than soils with cherry or oak leaf litter (Schwaner and Kelly, 2019). These differences represent a potential for increased storage of C in surface soil of forests with re-introduced American chestnut as the microbial community accumulates biomass in an N-limiting environment.

As discussed earlier, chestnut leaf litter is of higher nutritional value for aquatic macroinvertebrates than oak, so the replacement of oak by chestnut may increase macroinvertebrate activity, with potential consequences on population, community, and ecosystem levels (Smock and MacGregor, 1988; Section 2.3.2). Additionally, slow decaying chestnut wood may increase stream channel complexity as it replaces faster decaying species, providing additional habitat for fish and invertebrates (Ellison *et al.*, 2005).

American chestnut can hybridize with native chinquapins, which, like American chestnut, continue to persist within their former range in the presence of blight. We would not expect such hybrids to possess any greater invasive tendencies than pre-blight American chestnut-chinquapin hybrids, which were known to occur (Shaw *et al.*, 2012). However, a hybrid inheriting the trait of blight tolerance could potentially have a competitive advantage over blight-susceptible chinquapins. While blight-tolerant American chestnut would be sexually compatible with Asian and European species, and may be intentionally bred with these species by horticulturalists, we would not expect such hybrids to possess weedy characteristics, based on the lack of reported weediness of any non-native or hybrid *Castanea* in North America to date (Section 2.1.2).

American chestnuts can potentially offer unique ecological benefits in coal mine reclamation efforts, where their tolerance of acidic well-drained soils and lack of canopy competition may be beneficial to their early-successional establishment (Gilland and McCarthy, 2012; Skousen *et al.*, 2013; Bauman *et al.*, 2014). This could in turn help broader reclamation efforts on these severely degraded sites.

2.6 Propagation of *Castanea*

Chestnut can be propagated sexually by seed, and asexually by tissue culture and grafting. Each of these methods is well suited for different applications. Other asexual propagation methods, such as rooting of hardwood or softwood cuttings and layering, have been reported with limited success, but are not likely to play a significant role due to high labor requirements and low survival of propagules. If regulatory approval is granted, distribution of Darling 58 material will likely include a combination of pollen, scion wood for grafting, seed, and seedlings, with a focus on outcrossing to increase genetic diversity (Section 11.2).

2.6.1 Pollination and seed production

Under typical orchard conditions in open (unshaded) areas, American chestnut trees begin producing seed at an age of 4 to 6 years (Thor, 1978; Wang *et al.*, 2013). Production of female burs generally lags behind production of male catkins by about 2 years. Flowering of chestnuts in shaded or understory conditions may be substantially slower (Section 2.2.2).

Pollen production can be accelerated to less than one year for some trees in high light growth chambers (Baier *et al.*, 2012) or optimal greenhouse conditions. This technique can shorten intergenerational times for breeding, and has been used to produce the T1 (transgenic American x wild-type American) generation of trees: mature wild-type trees in orchards were hand pollinated with pollen produced on transgenic trees in growth chambers. Potted trees in growth chambers produce limited quantities of pollen, and pollen viability appears to decrease after long-term storage, so is not currently suitable for large scale seed production.

Chestnuts can be propagated by seed either through controlled pollination or open pollination. In controlled pollination, female flowers are bagged while immature to exclude wind borne pollen if other flowering chestnuts are present nearby. When female flowers mature, they are then hand pollinated, and the bag replaced. Pollen for controlled pollinations may be collected fresh from nearby trees, or if appropriate desiccation and storage techniques are employed, stored frozen for at least several months and then re-hydrated. Pollen storage allows for the use of pollen that was produced on indoor containerized plants throughout the year, and facilitates pollination of isolated trees where a particular type of pollen isn't otherwise available.

Fresh chestnut seeds may be shipped or stored temporarily unrefrigerated, but must be protected from desiccation, and typically require cold stratification before germination (Section 2.2.2). This may be achieved by storage at around 4°C for 60 – 90 days. Seeds may be sown directly in the field or started in containers after cold stratification. Seedlings typically grow rapidly and with good form, supported by the seed's supply of carbohydrates and nutrients.

2.6.2 Effective pollination distance for American chestnut

Chestnut is self-infertile, so trees will only produce seed when a sexually compatible, flowering tree is close enough to pollinate female flowers. Awareness of effective pollination distance is crucial in planning restoration plantings and seed production orchards. It can be used by managers to predict and control the hybridization of various chestnut species and varieties.

Effective pollination distance is the distance that pollen can travel and remain viable so that it can fertilize a female flower and produce a viable seed. Chestnut pollen is prone to desiccation and loses viability within as little as a few hours when air dried (Maynard, 1991), so effective pollination becomes increasingly unlikely as pollen spreads farther from its source. There are few peer-reviewed studies on the effective pollination distance for American chestnut, but reported practical applications from chestnut growers and results from related species are informative. There are several reports of effective pollination distance for American chestnut from informal and non-reviewed sources. These reports include both wind and insect pollination because they examine viable nut production success, regardless of how the pollen is vectored. Jacobs *et al.* (2011) report that trees need to be within 100 meters for successful pollination. Rutter (1990) states that “trees only 100 feet [30 m] apart will experience reduced pollination success, and trees 1000 ft. [300 m] apart are essentially reproductively isolated from one another.” He then defines isolation in the glossary: “isolation- when a tree is so far from others that they cannot pollinate it- 100 yards [90 m]”, which closely matches Jacobs *et al.* (2011).

A recent report on pollination of Japanese chestnuts in an orchard setting evaluated trees at 4, 8, 12, and 16 m from the pollen source, and found that pollination was highest for the trees at 4 m and decreased with distance. Maximum or isolation distances were not tested in this experiment (Nishio *et al.*, 2019). A report on European chestnut showed that fertilization rates dropped as distance increased, up to a maximum distance of 400 m (Villani and Eriksson, 1999).

To prevent Internal Kernel Breakdown (IKB) caused by crosses between Chinese chestnut and the Colossal hybrid, The Midwest Nut Producers Council recommends keeping these trees at least 90 m from each other to prevent pollination. According to Dr. Dennis Fulbright from Michigan State University:

"I would say we have evidence that pollen can move at least three quarters of a mile [1.2 km]. The Chinese chestnut trees are 40-years old, tall (35 – 40 feet) [10 – 12 m] and robust. I believe pollination is all about height of the trees as pollen from small, young trees does not fly very far. Once they get taller, it appears the pollen can take off and fly. This has surprised us and we are glad we checked this as pollen-isolated trees for study need to be planted further away, at least if downwind. Therefore, at least downwind, the pollen can fly more than 1000 m." (Personal communication)

To summarize Dr. Fulbright's observations, large (i.e. greater than 10 m tall) trees can send pollen much further than small ones. Anecdotal observations suggest it takes approximately 10 to 15 years under optimal conditions for American chestnuts to reach this height.

Data on pollination distance from agricultural settings may not be directly applicable to forest settings. Effective pollination distance will likely be reduced in a forest setting with higher tree density, as evidenced by studies that have shown increased pollination distances under silvicultural practices that decrease stand density (Smouse and Sork, 2004). This effect may be more pronounced for chestnut than other wind pollinated species, as chestnut flowers release pollen later in the season, when the canopy is filled with leaves, unlike trees such as oak and hemlock, which produce pollen before deciduous trees have fully leafed out (Paillet, 2002). Further experiments are needed to refine the effective pollination distance for chestnut in a forest setting.

If high pollination (and consequently high seed production) rates are desired, we recommend spacing compatible trees as close as possible. If pollination isolation is desired, we recommend standards that take into account tree height. For trees up to 6 m in height, a 400 m (~¼ mile) separation distance should effectively isolate trees: this is over the longest distance reported for isolation with the American chestnut. For trees substantially taller than 6 m, increasing the distance may provide additional assurance of isolation. Tree height can be maintained by pruning or coppicing.

2.6.3 Micropropagation by tissue culture

Both somatic embryos and meristem tissue of American chestnuts can be propagated via tissue culture (Maynard *et al.*, 2015; Andrade *et al.*, 2009). The shoot cultures can be multiplied, rooted either by *in vitro* or *ex vitro* protocols (Oakes *et al.*, 2016b), and then acclimated to greenhouse and field conditions. Micropropagation techniques allow for relatively rapid production of a few specific genotypes, but is labor intensive and costly. American chestnut trees produced from tissue culture have exhibited poor vigor and plagiotropic (angled, horizontal or branch-like) growth form as compared to seedling derived trees (Callahan, 2015), but this may be corrected by coppicing after establishment. Additionally, preliminary observations in our laboratory indicate that, during and after rooting, survival and vigor of chestnut plantlets decrease with length of time *in vitro*. Micropropagation has successfully been used to produce pollen donor trees for controlled pollinations and scion wood for grafts.

2.6.4 Grafting

Grafting has regularly been used to propagate desirable chestnut genotypes and speed up flowering times for selection programs (Thor, 1978). American and Asian chestnut species are

amenable to grafting using traditional techniques such as whip-and-tongue, cleft, and budding. However, graft union failure is not uncommon, both initially and delayed. Graft union failure may be a result of interspecific or interclonal incompatibility and/or poorly aligned vascular connection due to non-uniformly distributed phloem fiber bundles in chestnut species (Huang *et al.*, 1994; McKenna and Beheler, 2016).

The graft union is particularly susceptible to chestnut blight, which gains entry to the tree at wound sites. In our limited observations, chestnut blight can infect a graft union even when the scion, rootstock, or both are blight tolerant. The trait of blight tolerance is not conferred to a scion grafted onto a blight-tolerant rootstock, or vice versa, whether blight tolerance is achieved through transgenic expression of OxO, or by naturally resistant Asian species and hybrids.

Topwork grafting, or the grafting of scions onto the branches of an established tree, will likely play a role in restoration breeding. Scions may be collected from diverse wild trees and grafted onto mature trees in orchards, or blight-tolerant scions can be grafted into a mature wild tree to provide a source of pollen. If this grafted material flowers, it can effectively allow self-pollination, since flowers on the grafted branch would no longer be clonally identical to flowers elsewhere on the tree. Grafts have been observed to flower in the first growing season after grafting onto mature trees (McKenna and Beheler, 2016; personal observation).

3.0 Biology of *Cryphonectria parasitica* and chestnut blight

3.1 Chestnut blight and *Castanea*

Chestnut blight, caused by the ascomycete fungus *Cryphonectria parasitica* (formerly *Endothia parasitica*), is a stem canker disease affecting all *Castanea* (chestnut) species. (In this document we generally use the term “blight” when referring to the broader interaction between pathogen and host, and specifically use “*C. parasitica*” or “blight fungus” when referring to the pathogen as an independent organism; see Section 6.3 for further discussion of terminology.) Asian chestnut species (*Castanea mollissima* and *C. crenata*) are generally regarded as blight resistant, though they can develop blight symptoms under certain circumstances. American chestnut is highly susceptible. North American chinquapin species are somewhat less susceptible. European chestnut is susceptible, though less so than American chestnut (Graves, 1950; Anagnostakis and Hillman, 1992).

Chestnut blight was first identified in 1904 in what is now the Bronx Zoo, then the New York Zoological Park (Merkel, 1905). *C. parasitica*, the causal agent, was later found to be endemic to Japan and China, and researchers concluded that the initial introduction of chestnut blight to North America occurred by importation of Asian chestnuts as nursery stock, probably from Japan, via the port of New York (Shear *et al.*, 1917; Anagnostakis, 1987; Milgroom *et al.*, 1996).

The blight resistance of Asian chestnut species is presumably due to a shared environment in which the trees and fungus co-evolved for millennia. However, Chinese chestnut cannot be considered immune under all circumstances. Chestnut blight has been observed as a regular occurrence on Chinese chestnut in apparently wild stands in China (Steiner *et al.*, 2017), and chestnut blight is regarded in China as an important disease in orchards (Yan *et al.*, 2007; Tarcali and Radocz, 2009). Nevertheless, during the past century several significant efforts have been made to hybridize Chinese chestnut with American chestnut, with the hope of capturing the blight resistance trait(s) evident in some of the Chinese species (Section 3.3.2).

The chestnut blight epidemic destroyed the populations of American chestnut and chinquapins throughout their natural range (Paillet, 1993). The recognizable impacts of blight upon chinquapin have not been as spectacular as those upon American chestnut because the stature, geographical range, and initial ecological and economic importance of chinquapin were less prominent (Johnson, 1987; Payne *et al.*, 1994), however the lingering effects are important. Ozark chinquapin especially was a prominent overstory tree in parts of its relatively small range in the Ozark plateau, where it provided ecosystem services much as the American chestnut did farther east (Paillet, 1993; Paillet and Cerny, 2012). The remaining chinquapin specimens among the forest understory rarely reach maturity, but still may act as reservoir populations for the blight pathogen.

In addition to members of the chestnut genus, *C. parasitica* is able to survive and reproduce on other hardwood species. There are many reports of the fungus as a saprophyte to mild parasite on multiple oak species (Gravatt, 1952; May and Davidson, 1960; Gruenhagen, 1965; Nash and Stambaugh, 1987; Davis *et al.*, 1997; Frigimelica and Faccoli, 1999). Other reported hosts include maple, hickory, American beech, American hornbeam (*Carpinus caroliniana*), American hophornbeam (*Ostrya virginiana*), staghorn sumac (*Rhus typhina*), and tulip tree (Stipes *et al.*,

1978). Additionally, some eucalypts appear to be susceptible to infection by *C. parasitica* (Old and Kobayashi, 1988). While occasionally causing cankers or swollen butt, the fungus does not appear to be a significant disease threat to any North American species outside the genus *Castanea*. However, due its ability to persist as a saprophyte on alternate hosts, *C. parasitica* has become permanently established in eastern forests, even as chestnut trees have become relatively rare. The fungus is not likely to dissipate to the extent that susceptible American chestnut could be sustainably repopulated without becoming infected.

3.2 Chestnut blight infection

Initial infection by *C. parasitica* occurs most commonly at branch points, where natural movement creates small gaps or wounds in the bark (Davelos and Jarosz, 2004), but may enter at any wound site. The fungus produces mycelia (threadlike filaments) that fan out through nearby tissues (Murrill, 1906; Griffin, 1986; Newhouse, 1990). In response, the tree forms a raised or sunken canker (Griffin, 1986; McManus *et al.*, 1989). A sunken canker occurs as a result of necrosis and the collapse of bark tissue, while a raised canker has both sunken areas where the bark tissue has been killed, and swollen areas where the tree has grown tissue in reaction to the mycelial fans (Griffin, 1986; Rigling and Prospero, 2018). The fungus typically penetrates into the vascular cambium and inner phloem which, when killed by fungal-secreted oxalate, cuts off the transport of nutrients and water throughout the portion of the tree above the canker (Murrill, 1906; Griffin, 1986; McManus *et al.*, 1989; Rigling and Prospero, 2018). As a result, the leaves of the tree wilt, turn brown, and eventually the stem or trunk area above the infection dies (Merkel, 1905; Griffin, 1986; Rigling and Prospero, 2018).

Secretion of oxalic acid (OA) is one of the primary pathogenesis mechanisms employed by *C. parasitica*. This is a strong organic acid found in a number of plants and fungi, and it is a general toxin to most organisms (Svedružić *et al.*, 2005; Williams *et al.*, 2011; Chakraborty *et al.*, 2013). OA lowers pH and binds calcium from plant cells, both of which weaken and kill chestnut stem tissue, which is then broken down by polysaccharide-degrading enzymes (McCarroll and Thor, 1978; McCarroll and Thor, 1985), allowing the necrotrophic *C. parasitica* to spread by consuming dead tissue. OA also suppresses natural plant defense responses including phenol oxidases and the oxidative burst, further facilitating continued infection by pathogenic fungi (Cessna *et al.*, 2000; Donaldson *et al.*, 2001). However, normal production of OA is not essential for initiating infections or continued survival of *C. parasitica*, as demonstrated by both naturally hypovirulent and engineered strains of the blight fungus that produce dramatically lower OA quantities, both of which can still initiate infections on chestnut stems but don't form the same damaging cankers as typical virulent strains (Havir and Anagnostakis, 1983; Chen *et al.*, 2010). Additionally, OA production varies substantially by the same strains of *C. parasitica* grown in different nutrient conditions, and OA production is not directly correlated to growth rates (mycelial production) of the fungus (Havir and Anagnostakis, 1983; Bennett and Hindal, 1989), both of which indicate this compound is not essential to survival of the fungus.

Chestnut species typically respond to the disease with vegetative sprouting from adventitious buds below the infection (Merkel, 1905) or from the root collar of the tree (Griffin, 1986; Paillet, 1988; Newhouse, 1990; Rigling and Prospero, 2018). These shoots are eventually attacked by the fungus themselves, resulting in a cyclical pattern of resprout and shoot death. Individual chestnut

clones may survive for decades in this manner, but blight infection does reduce survival, especially when combined with competition and/or abiotic stress (Griffin *et al.*, 1991; Parker *et al.*, 1993).

3.3 Existing methods for addressing chestnut blight

Numerous means of treating or controlling chestnut blight have been attempted in the years since chestnut blight was discovered on American chestnuts. None have achieved any meaningful level of control in a forest setting.

3.3.1 Biocontrol of chestnut blight

One method for controlling blight by addressing the pathogen rather than the host is known as hypovirulence, or a state of reduced virulence in isolates of *C. parasitica* caused by infection with a double-stranded RNA hypovirus (Dawe and Nuss, 2001; Choi *et al.*, 2012). Hypovirulent cankers expand slowly if at all, and the tree can generally heal and outgrow these cankers. Hypovirulent fungus can be applied to an existing canker as a treatment; the fungus on the tree is converted to the hypovirulent state upon fungal anastomosis (Anagnostakis, 1982). Anastomosis is the merging of compatible fungal hyphae that come into physical contact, allowing the sharing of genetic and other material.

In Europe, hypovirulence spread naturally through *C. parasitica* populations on European chestnut, augmented by deliberate inoculations as a biocontrol of chestnut blight in orchards (Milgroom and Cortesi, 2004). In some areas of Europe, most cankers appear to be healing as a result of hypovirulence, and blight-induced mortality is low, while in other areas, few healing cankers are seen. Additionally, blight-induced mortality is high in some areas with many healing cankers, indicating that the presence of hypovirulence does not always significantly retard disease progress (Milgroom and Cortesi, 2004).

Not all pairs of fungal strains are able to anastomose and transfer the virus; this limitation is termed vegetative incompatibility, and is regulated by at least six loci in the *C. parasitica* genome (Choi *et al.*, 2012). Fungal strains that are compatible are said to share a vegetative compatibility (vc) group. Hypovirulence as a biocontrol strategy has been limited in North America by the complex system of vegetative incompatibility on this continent (MacDonald and Double, 2005). An increasing number of vc groups in the European fungal population may also jeopardize the use of hypovirulence there (Robin and Heiniger, 2001). Recent biotechnological advances have demonstrated that it is possible to engineer *C. parasitica* to enable hypovirus transmission between multiple vc groups (Stauder *et al.*, 2019). Such engineered hypovirulent strains of *C. parasitica* are currently being investigated under APHIS permits.

With the exception of American chestnut in Michigan (outside chestnut's original native range), where hypovirulence has spread without human assistance and has increased survival of many large trees (Brewer, 1995), hypovirulence does not appear to spread naturally in North America (Milgroom and Cortesi, 2004; Anagnostakis, 1982). Even where natural spread does not occur, hypovirulent treatment has some effectiveness as an intentional inoculation of individual infected trees or even individual cankers, but this technique is relatively labor intensive, and not practical on a landscape scale for forest trees or even for very large orchard trees which may develop cankers out of reach (Heiniger and Rigling, 2009).

Another treatment for individual cankers is known as a soil compress (Weidlich, 1978), also known as “mud packing” (The American Chestnut Foundation, 2019a). This technique involves application of local field soil to a canker and the surrounding tissues, which is left in place for two or more months. A compress of compost rather than field soil was also found to be effective (Groome *et al.*, 2001). The mode of action is likely one or more naturally occurring soil fungi or bacteria, which can be antagonistic to *C. parasitica*. Application of commercial formulations of antagonistic microorganisms such as the bacteria *Bacillus subtilis* may also reduce canker severity (Murolo *et al.*, 2019).

These techniques are only effective in treating individual cankers, and chestnut trees will inevitably develop new infections throughout the tree. However, hypovirulent and soil compress treatments may be useful for protecting a limited number of valuable trees, at least temporarily. One particular case suggested by Weidlich (1978) is for protecting the graft union of grafted trees, which is particularly susceptible to blight.

3.3.2 Breeding programs for blight resistance

Horticulturalists with the USDA began breeding American chestnut with imported Asian and European chestnut species for nut production as early as 1894, before the introduction of chestnut blight (Van Fleet, 1914). When blight began killing trees in these orchards, the focus narrowed to a few lines derived from crosses of American chestnut with European chestnut, Chinese chestnut, Japanese chestnut, and Allegheny chinquapin. In 1922, breeding work for blight resistance began under the USDA’s Office of Forest Pathology, with American chestnut and Chinese chestnut hybrids selected for timber-type growth form and blight resistance (Burnham *et al.*, 1986). From that effort several promising first-generation *C. mollissima* x *C. dentata* hybrid trees were generated, including the ‘Clapper’ tree, which for several decades did exhibit good growth and slower progression of chestnut blight, yet eventually did die from it. The USDA breeding program was abandoned in 1960 (Diller, 1965).

In 1921, Arthur Graves started planting trees on land he owned, and became one of the most prolific early breeders, crossing American chestnut with a variety of Asian chestnut species. His land was deeded to the Connecticut Agricultural Experiment Station in 1950, which continued to breed chestnuts for blight resistance, producing the ‘Graves’ tree and its associated genetic lines, also derived from Chinese x American chestnut hybridization (Graves, 1940; Anagnostakis, 2012).

None of these early programs succeeded in producing a fast growing, timber-type (tall and straight, a hallmark of the American chestnut) tree with good blight resistance; every candidate fell short in at least one of these respects (Diller and Clapper, 1969; Burnham *et al.*, 1986; Anagnostakis, 2012).

The American Chestnut Foundation (TACF) was founded in 1983 as a coalition of plant scientists and laypersons interested in the preservation of the species. Soon thereafter, three of the foundation’s prominent scientists published an extensive paper describing the foundation’s breeding plan (Burnham *et al.*, 1986). The Burnham plan proposed a systematic program of backcrossing hybrid trees with pure American chestnut trees, selecting for blight resistance and American phenotype at each step. The backcross method, already used extensively in agriculture, integrates the principles of Mendelian and quantitative genetics. Burnham proposed to backcross

F1 hybrids with American trees for 3 generations, which, after extensive tree selection, should achieve the American phenotype while maintaining the quantitative blight resistance genes from the Chinese parent in a heterozygous state. The next step would be to intercross these BC₃ trees with each other to produce the BC₃F₂ generation. Crossing selected BC₃F₂ trees with each other produces BC₃F₃ (also called B₃F₃) trees, which would be distributed for restoration plantings. Selected for only the most blight-resistant individuals in each generation, the BC₃F₃ trees would, the authors predicted, stack all the resistance genes in a homozygous state and have functional blight resistance matching the Asian species.

TACF's breeding program remains active, and has begun to implement genomic selection to improve the speed and efficiency of candidate selection (Steiner *et al.*, 2017). BC₃F₃ trees are currently planted in TACF's research and outreach orchards, but are not widely available to the general public. Recent inoculation screening generally shows that backcross trees have intermediate levels of resistance between the original Chinese parent and wild-type American chestnut (Cipollini *et al.*, 2017; Clark *et al.*, 2019), and therefore may have lost one or more resistance genes during breeding. The original plan was based on a hypothesis of two resistance genes (Burnham *et al.*, 1986), but subsequent observations suggested that there are at least 3 – 6 separate genes for the quantitative blight resistance in the Chinese chestnut (Hebard, 2012). The most recent data reveal a correlation between blight resistance and proportion of Chinese genome in backcross, which suggests inheritance of the blight resistance trait is polygenic rather than controlled by a few discrete genes (Westbrook *et al.*, 2019b). Further breeding, selection, and genomic analysis are being done in an attempt to optimize blight resistance inherited from Chinese parent trees and incorporate resistance to *Phytophthora* root rot, while maintaining American chestnut phenotypes (Steiner *et al.*, 2017; Westbrook *et al.*, 2019b).

A natural effect of the breeding process is linkage drag. By selecting for resistance genes, thousands of Chinese chestnut genes that are not involved with resistance, but which are located close to each of the resistance loci, are also part of the BC₃F₃ genome (Nelson *et al.*, 2014), possibly resulting in the loss of alleles from the American ancestors.

Apart from the backcross program, various chestnut hybrids have been produced that are commercially available today including Dunstan Chestnut (patented *C. dentata* x *mollissima* hybrid, marketed by Chestnut Hill Tree Farm), ECOS American hybrid chestnut and "Timburr chestnut" (*C. dentata* x *mollissima* hybrid, marketed by Oikos Tree Crops as "the best selection to use for bringing back the chestnut"), Badgersett Hybrid Chestnut (complex cross *C. dentata* x *mollissima* x *sativa*, marketed by Badgersett Research Corporation), Timber Hybrids (complex *C. dentata* x *mollissima* x *sativa* crosses marketed by Empire Chestnut Company), and Miller Wild life Seguin Hybrid Chestnut (*C. seguinii* x *dentata* hybrid, marketed by Oikos Tree Crops). These trees were all produced by conventional breeding practices and can be planted anywhere in the United States without restriction, despite documented risks that can occur with some hybrid crosses (Section 2.1.2).

3.3.3 Mutational breeding for blight resistance

Starting in the 1950s and proceeding into the 1970s, W. Ralph Singleton, in collaboration with Albert Dietz, exposed chestnut seeds to radiation, with the hope of inducing a mutation that would confer blight resistance to the resulting tree (Dietz, 1978; Burnworth, 2002; Curry, 2014).

The technique had been used by Singleton and others on a wide variety of crops, in many cases resulting in disease resistance or other desirable traits. The products of mutational breeding were and are not regulated by any governmental agency in the United States. Chestnut seeds collected by Dietz were irradiated at Brookhaven National Laboratory and other facilities, and over 10,000 trees were planted in orchards ranging over 7 states (Dietz, 1978), but no trees were reported to have any confirmed resistance to chestnut blight.

3.3.4 Chemical control of chestnut blight

Though numerous chemical controls have been attempted over the years, no fungicide has been shown to be a practical option for controlling blight on a wide scale (Merkel, 1905; Rigling and Prospero, 2018). In Europe, fungicides are occasionally used in managed (orchard or nursery) settings, especially to protect graft unions (Milgroom and Cortesi, 2004; Döken, 2009; Trapiello *et al.*, 2015). Unpublished and anecdotal reports have also noted that commercially available fungicides (which are labeled for use on chestnut) may help control blight on individual trees (Penn State, 2014; AgBio Inc., 2019). However, chemical control of chestnut blight is not feasible on a landscape scale due to cost, phytotoxicity, evolved resistance in the pathogen, and restrictions placed on the use of fungicides in forest settings (Rigling and Prospero, 2018).

4.0 Oxalate oxidase (OxO) in plants

4.1 Background and properties of OxO

Oxalate oxidase (OxO) belongs to a very diverse family of genes for germin and germin-like proteins (GLPs) found in all plants (Dunwell et al., 2008). OxO facilitates conversion of oxalates into hydrogen peroxide and carbon dioxide (Figure 4.1a; Laker et al., 1980; Woo et al., 2000). Carbon dioxide is a natural product of organismal metabolism which is used by plants during photosynthesis, and hydrogen peroxide is widely produced in plants for a variety of purposes (see Section 4.3 for a detailed discussion of hydrogen peroxide). OxO and a related enzyme called superoxide dismutase comprise a GLP subgroup consisting of hydrogen peroxide-generating enzymes (Dunwell et al., 2008).

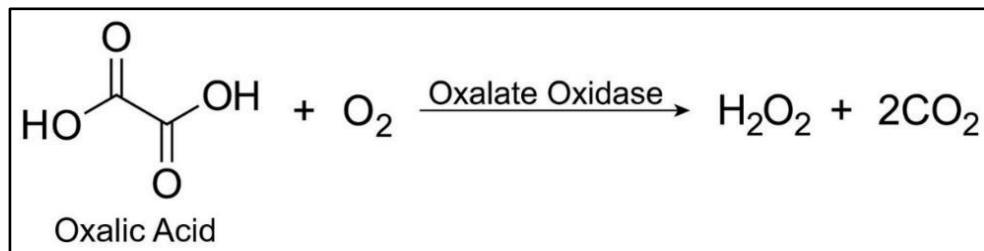


Figure 4.1a. Oxalate oxidase catalyzes the degradation of oxalic acid into hydrogen peroxide and carbon dioxide.

OxO was first described in 1911 by Zaleski and Reinhard from powdered wheat grains (Davoine et al., 2001; Zaleski and Reinhard, 1911; see translation of introduction attached to article PDF), and was subsequently identified from moss and further described in 1927 (Houget et al., 1927; see translation of abstract attached to article PDF). Subsequently, OxOs were characterized from wheat, barley, corn, rye, rice, and oat (Lane et al., 1993; Lane, 2002). These true cereal OxOs were dubbed germin OxOs to distinguish them from OxOs found in other plants (Lane, 2002). OxOs are not limited to true cereals: OxO isoforms have been found in a variety of other cultivated and wild plants (Tables 4.2a and b), including mosses (Laker et al., 1980), shellflower (Volk et al., 2002), beet (Obzansky and Richardson, 1983), banana (Anjum et al., 2014), azalea (Sakamoto et al., 2015), spinach (Lane, 2002), switchgrass (Figure 4.2a), and several other organisms including bacteria and fungi.

Germin OxO is a primary constituent of germinating wheat embryos where it is glycosylated to the cell wall, providing hydrogen peroxide necessary for cross-linking reactions that help in cell wall expansion and lignification (Lane et al., 1993; Lane, 2002). This hydrogen peroxide generation via germin OxO allows for tissue remodeling as the wheat embryo uptakes water and undergoes the fastest growing period of its life cycle (Lane et al., 1991; Lane et al., 1993; Caliskan et al., 2004). Germins and GLPs may also contribute to cell wall structural reinforcements even in the absence of specific oxalate oxidase activity (Schweizer et al., 1999). This physical barrier may directly affect the initiation of fungal infection, and also provides time for slower transcription-based plant defense responses like lytic enzymes, general antimicrobial proteins, and phytoalexins to begin working (Brisson et al., 1994; Bolwell and Wojtaszek, 1997).

Calcium oxalate is a naturally stable storage molecule for calcium ions (Ca^{2+}). By using OxO to degrade oxalate, plants can access stored Ca^{2+} for periods of stress and senescence (Davoine *et al.*, 2001; Volk *et al.*, 2002). In ryegrass (*Lolium perenne*), calcium oxalate crystals are stored mainly in the vacuoles and are associated with senescence of leaf sheaths: as leaf sheaths age, OxO activity increases and breaks down stored calcium oxalate. The increasing concentration of Ca^{2+} and H_2O_2 products signal induction of senescence in the leaf sheaths, remobilizing C and N nutrients to growing tissues (Davoine *et al.*, 2001).

Volk *et al.* (2002) looked at calcium oxalate crystal formation and breakdown in *Pistia stratiotes* leaves based on calcium availability. When grown in calcium-starved environments, calcium oxalate crystals in *Pistia stratiotes* disappeared, while plants grown in calcium-rich environments had large crystal formations in their tissues. Tagging of OxO enzymes through transmission electron microscopic analysis revealed high concentrations of OxO in tissues in calcium-stressed environments, and relatively low concentrations of OxO in calcium-rich environments. Increased expression of OxO in calcium-starved plants suggests one role of OxO is mediating cytosolic and apoplastic Ca^{2+} concentrations (Volk *et al.*, 2002).

Some types of wood rot fungi also produce oxalic acid as part of the wood decay process. This mechanism is not pathogenic, but rather allows the fungi to degrade and consume dead tissue, and also possibly to tolerate excess copper (another cation that can be bound to oxalate) (Clausen and Green, 2003). Since oxalic acid can be toxic at high concentrations, these wood rot fungi also have an enzymatic mechanism to break down this acid (oxalate decarboxylase; similar in result to oxalate oxidase) (Hastrup *et al.*, 2012).

In addition to its widespread presence in plants, OxO is being studied and employed for various medical, nutritional, and industrial applications (Hu, J. *et al.*, 2015). Most directly, medical conditions resulting from high oxalate (i.e. hypoxaluria) can potentially be treated with OxO in various forms (Pundir and Verma 1993; Allison and Sidhu, 2001; Cowley and Li, 2017). A novel treatment of pancreatic cancer cells involves high doses of ascorbate (vitamin C), which shows promise for treating this type of cancer, but can result in dangerous oxalate buildup: OxO treatments could prevent this side effect (Du *et al.* 2010; Goodwin *et al.*, 2017). OxO can also be immobilized on medical devices such as ureteral stents to prevent encrustation by calcium oxalate (Malpass *et al.*, 2002; Mellman, 2007). Putative nutritional benefits have been reported in transgenic plants by integrating an oxalic acid breakdown pathway in a food product. Oxalate decarboxylase was transformed into soya bean (*Glycine max*) and grass pea (*Lathyrus sativus*) to reduce oxalic acid concentrations in edible tissues, thus reducing the likelihood of calcium oxalate crystals forming in kidneys of people eating these products (Kumar *et al.*, 2016). This is a unique case; most transgenic applications of OxO and other oxalic acid-degrading enzymes in plants are used primarily for pathogen tolerance, and most proposed medical applications don't involve transgenic plants.

In addition to medical applications, OxO has been proposed as a component of food packaging films, where it could act as a preservative by scavenging both oxygen and oxalic acid, while releasing protective carbon dioxide (Winestrand *et al.*, 2013). OxO is part of a multi-enzyme process that could facilitate the efficient use of glycerol in biofuel cells (Arechederra and Minteer, 2009; Hickey *et al.*, 2014). Finally, oxalate crystals can be problematic on machinery in the

paper/pulping industry, so OxO and similar enzymes could potentially provide a non-toxic means of preventing this buildup or scaling (Sjöde *et al.*, 2008; Cassland *et al.*, 2010).

As far as we are aware, none of these properties or applications of OxO would represent novel plant pest risks. On the contrary, most represent mechanisms that are seen as safer or more natural than synthetic alternatives.

4.2 Presence of native OxO in plants and other organisms

Oxalate oxidase has been identified and characterized in a variety of organisms: cereal grains as described in Section 4.1, various unrelated dicots, lower vascular plants, and even bacteria and fungi. These include plants cultivated for food (Table 4.2a) and wild organisms (Table 4.2b). Both of these tables contain examples of OxO genes or enzymatic activity, primarily from published reports in either academic journals or National Center for Biotechnology Information (NCBI) databases.

OxO activity was observed in our lab using a histochemical assay (Section 7.4.1) for switchgrass (*Panicum virgatum*), one of the wild plants in Table 4.2b. Switchgrass seeds were obtained from Prairie Nursery (Westfield, WI) in 2016. The assay showing OxO activity was performed in the Powell lab by D. Decker and D. Matthews (Figure 4.2a). The presence of an OxO gene or enzyme activity in switchgrass has not been previously published.

The variety of plants and other organisms in which OxO activity has been identified and published suggest that OxO activity is widespread in nature, and is likely found in many other foods and wild plants as well.

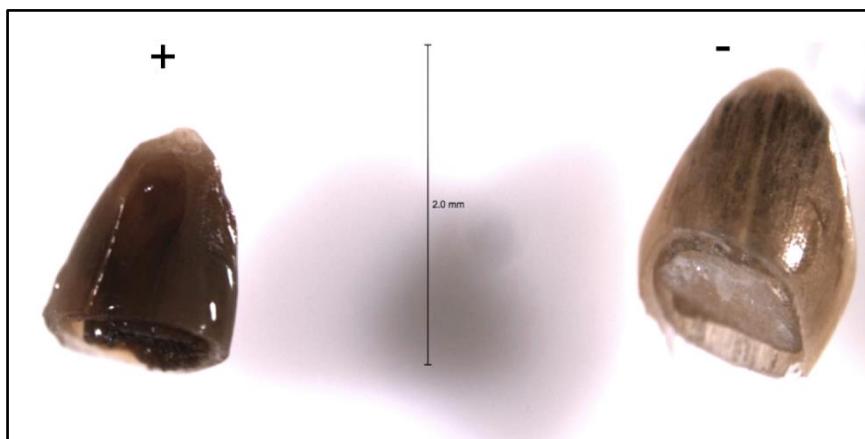


Figure 4.2a. Oxalate oxidase activity assay in seeds of switchgrass (*Panicum virgatum*). Dark staining in “+” reaction (left) and lack of staining in the “-” reaction (right) indicates OxO activity (Matthews *et al.*, unpublished). Scale bar in center is 2.0 mm.

Table 4.2a. Cultivated food plants with identified oxalate oxidase gene or enzyme activity.

| CULTIVATED FOOD PLANTS | | |
|------------------------|--------------------------------|---------------------------------|
| Common Name | Scientific Name | Reference |
| Peanut | <i>Arachis hypogaea</i> | (Wang <i>et al.</i> , 2010) |
| Oat | <i>Avena sativa</i> | (Lane <i>et al.</i> , 1991) |
| Sugar beet | <i>Beta vulgaris</i> | (Arnon and Whatley 1954) |
| Tea | <i>Camellia sinensis</i> | (Fu <i>et al.</i> , 2018) |
| African oil palm | <i>Elaeis guineensis</i> | (Rusli <i>et al.</i> , 2015) |
| Finger millet | <i>Eleusine coracana</i> | (Akbar <i>et al.</i> , 2018) |
| Strawberry | <i>Fragaria ananassa</i> | (Dahiya <i>et al.</i> , 2010) |
| Barley | <i>Hordeum vulgare</i> | (Sugiura <i>et al.</i> , 1979) |
| Tomato | <i>Lycopersicon esculentum</i> | (Sun <i>et al.</i> , 2019) |
| Banana | <i>Musa paradisica</i> | (Anjum <i>et al.</i> , 2014) |
| Rice | <i>Oryza sativa</i> | (Carrillo <i>et al.</i> , 2009) |
| Scarlet runner bean | <i>Phaseolus coccineus</i> | (Chipps <i>et al.</i> , 2005) |
| Date palm | <i>Phoenix dactylifera</i> | (NCBI, 2018c) |
| Peach & Apricot | <i>Prunus spp.</i> | (Liang <i>et al.</i> , 2010) |
| Rye | <i>Secale cereale</i> | (Lane, 2000) |
| Sorghum | <i>Sorghum bicolor</i> | (Satyapal and Pundir, 1993) |
| Spinach | <i>Spinacia oleracea</i> | (Laties, 1950) |
| Cacao | <i>Theobroma cacao</i> | (Gesteira <i>et al.</i> , 2007) |
| Wheat | <i>Triticum aestivum</i> | (Lane <i>et al.</i> , 1993) |
| Corn | <i>Zea maize</i> | (Vuletić and Šukalović, 2000) |

Table 4.2b. Wild plants and other organisms with identified oxalate oxidase gene.

| WILD AND ORNAMENTAL PLANTS | | |
|-----------------------------|--------------------------------------|----------------------------------|
| Common Name | Scientific Name | Reference |
| Goatgrass | <i>Aegilops tauschii</i> | (NCBI, 2017) |
| Spiny amaranth | <i>Amaranthus spinosus</i> | (Goyal <i>et al.</i> , 1999) |
| Ramie | <i>Boehmeria nivea</i> | (Xuxia <i>et al.</i> , 2012) |
| Bougainvillea | <i>Bougainvillea spectabilis</i> | (Srivastava and Krishnan, 1962) |
| Stiff brome | <i>Brachypodium distachyon</i> | (NCBI, 2018b) |
| Rubber bush | <i>Calotropis procera</i> | (Freitas <i>et al.</i> , 2017) |
| Mexican tea (epazote) | <i>Chenopodium ambrosioides</i> | (Nagahisa and Hattori, 1964) |
| Insulin plant | <i>Costus pictus</i> | (Sathishraj and Augustin, 2012) |
| Common sunflower | <i>Helianthus annuus</i> | (Maksoud, 1996) |
| Three-cornered hypnum moss | <i>Hypnum triquetrum</i> | (Houget <i>et al.</i> , 1927) |
| Various mosses (12 species) | <i>Hylocomium</i> spp. and others | (Datta and Meeuse, 1955) |
| Perennial ryegrass | <i>Lolium perenne</i> | (Davoine <i>et al.</i> , 2001) |
| White lupin | <i>Lupinus albus</i> | (Wojtaszek <i>et al.</i> , 1997) |
| Common ice plant | <i>Mesembryanthemum crystallinum</i> | (Michałowski and Bohnert, 1992) |
| Switchgrass | <i>Panicum virgatum</i> | Figure 4.2a |
| Castor bean | <i>Ricinus communis</i> | (NCBI, 2018a) |
| Azalea | <i>Rhododendron mucronatum</i> | (Sakamoto <i>et al.</i> , 2015) |
| Wild einkorn wheat | <i>Triticum urartu</i> | (NCBI, 2013) |
| Narrowleaf cattail | <i>Typha angustifolia</i> | (Du <i>et al.</i> , 2018) |

| FUNGI AND BACTERIA | | |
|----------------------|------------------------------------|--------------------------------|
| | | |
| Abortiporus mushroom | <i>Abortiporus biennis</i> | (Grąż <i>et al.</i> , 2016) |
| White rot fungus | <i>Ceriporiopsis subvermispora</i> | (Aguilar <i>et al.</i> , 1999) |
| Mycorrhizal fungus | <i>Laccaria bicolor</i> | (Mäkelä <i>et al.</i> , 2010) |
| Endophytic bacterium | <i>Ochrobactrum intermedium</i> | (Kumar and Belur, 2016) |
| Split-gill mushroom | <i>Schizophyllum commune</i> | (NCBI, 2016a) |
| Dwarf bunt fungus | <i>Tilletia controversa</i> | (Vaisey <i>et al.</i> , 1961) |
| Dermatophytic fungus | <i>Trichophyton rubrum</i> | (NCBI, 2016b) |
| Bacterium | <i>Pseudomonas</i> sp. Ox-53 | (Koyama, 1988) |

4.3 Mechanisms of OxO and hydrogen peroxide (H_2O_2) in plant defense

Germin OxOs and OxO isoforms can increase fungal pathogen resistance in plants through several possible means. The most direct is the detoxification of oxalate (oxalic acid), which is a virulence factor of *Cryphonectria parasitica* as described in Section 3.2. Many other fungal pathogens are also known to secrete oxalic acid (OA) as a pathogenicity factor, including *Botrytis cinerea* (Sun *et al.*, 2019), *Cristulariella pyramidalis* (Kurian and Stelzig, 1979), *Mycena citricolor* (Rao and Tewari, 1987), *Sclerotinia sclerotiorum* (Noyes and Hancock, 1981), *Sclerotium cepivorum* (Stone and Armentrout, 1985), and *Septoria musiva* (Liang *et al.*, 2001), and other fungi (Livingstone *et al.*, 2005). Given the diversity of pathogens that produce OA, it is not surprising that many plants have endogenous mechanisms for tolerating or degrading this toxin (Sections 4.2 and 5.4). Oxalic acid as secreted by these pathogens chelates and removes calcium from the host plant's cell walls and acidifies the ambient tissue, killing plant cells, inhibiting lignin formation, and allowing the mycelium to progress through the necrotic tissue (Anagnostakis, 1987; Cessna *et al.*, 2000; Livingstone *et al.*, 2005; Welch *et al.* 2007). Cereal crops and other monocots that express OxO don't tend to be susceptible to these OA-producing pathogens (Section 5.3). See Section 5 for a discussion of OA and OxO in the broader context of plant defenses against pathogens, and Section 6.3 for a more specific discussion of OxO in Darling 58 chestnut.

In addition to degradation of OA, there are three other mechanisms by which OxO has been hypothesized to help defend plants against fungal pathogens, all of which involve the hydrogen peroxide (H_2O_2) byproduct of OA degradation. These include direct microcidal activity of H_2O_2 , hypersensitive cell death or other plant defense responses induced by H_2O_2 , and cell wall lignification facilitated by H_2O_2 (Lane 2002; He *et al.*, 2013; Molla *et al.*, 2013). Direct antimicrobial properties of H_2O_2 in plants depend strongly on concentration and location of the H_2O_2 (Baldry, 1983; Peng and Kuc, 1992), while induced plant defense responses may not require H_2O_2 to be in immediate contact with the pathogen (Levine *et al.*, 1994; Neill *et al.*, 2002).

This subsection describes H_2O_2 in plants generally and from endogenous oxalate oxidase sources; further details on OxO-generated H_2O_2 in the context of transgenic chestnuts and potential plant pest risks are discussed in Section 6.3.2.

4.3.1 H_2O_2 production in plants

H_2O_2 and other reactive oxygen species (ROS) are naturally produced by several mechanisms in all plants (and some fungi) in response to various stimuli including pathogen infection and abiotic stresses (Orozco-Cardenas and Ryan, 1999; Demidchik, 2015). Endogenous mechanisms for H_2O_2 production include peroxidases, NADPH oxidase, and oxalate oxidase (Wojtaszek, 1997; He *et al.*, 2013). In the case of oxalate oxidase, OA is required for H_2O_2 production, which would spatially and temporally limit H_2O_2 concentrations (Section 4.3.3). Multiple experiments on plants transformed specifically to continually produce H_2O_2 have concluded that continuous moderate concentrations of H_2O_2 are not harmful to the plant, likely because it is inherently short-lived and scavenging mechanisms break it down before it reaches harmful concentrations (Orozco-Cardenas and Ryan, 1999; Ramputh *et al.*, 2002; Asselbergh *et al.*, 2007).

4.3.2 Effects of H₂O₂ in plant defense responses

As noted above, effects of H₂O₂ depend on concentration and location within the plant: it can facilitate lignification and cross-linking of cell walls to physically strengthen barriers to infection, it can act as a signaling molecule to stimulate other plant defense pathways, it can have direct antimicrobial activity, and it can even kill plant cells as part of the hypersensitive response, preventing the spread of biotrophic pathogens that rely on living plant tissue (Lane, 1994; Thordal-Christensen *et al.*, 1997; Yoda *et al.*, 2003; Svedružić *et al.*, 2005). Thus H₂O₂ is often beneficial to plants, though the death of plant cells is a trade-off that is not directly helpful to the plant (especially when responding to necrotrophic pathogens such as *C. parasitica*), so sometimes H₂O₂ can exacerbate damage along with stimulating necessary responses (Van Breusegem *et al.*, 2001).

In response to pathogen infection, many plants exhibit an oxidative burst, which is a rapid production of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals (Wojtaszek, 1997). Hydrogen peroxide and other ROS have been found to play an important role in plant defense responses (Section 4.1.2; Wojtaszek, 1997). In low concentrations, H₂O₂ can act as one of the primary transducers of general plant defense responses as it is rapidly diffusible (Lane, 1994; Thordal-Christensen *et al.*, 1997). H₂O₂ is transmitted through the transpiration stream of plants upon wounding, eliciting this defense response (Lane, 1994; Thordal-Christensen *et al.*, 1997; Lane, 2000). Increased concentrations of H₂O₂ in pathogen-stressed tissues undergoing an oxidative burst facilitate the modification of plant cell walls through peroxidase-catalyzed cross-linking of polymers. The rapid oxidative cross-linking of these polymers in the cell wall creates a physical barrier much more resistant to fungal pathogen invasion than normal cell wall structure (Brisson *et al.*, 1994). These heavily lignified cells can serve one final purpose in the defense response: their tough, less digestible cell walls may act as a trap, not allowing the fungal pathogen to spread easily outside of areas destined for programmed cell death (Brisson *et al.*, 1994). ROS may also inhibit fungal growth directly, or secondarily generate reactive free radicals that are antimicrobial (Peng and Kuc, 1992). At the highest concentrations of ROS, the host's plant cells are locally triggered to undergo programmed cell death via a hypersensitive response (Thordal-Christensen *et al.*, 1997; Brisson *et al.*, 1994; Delisle *et al.*, 2001).

4.3.3 Spatial and temporal distribution and limitations of H₂O₂

Since H₂O₂ production is widespread in plants and fungi, and since too much of it can be detrimental to living cells, both plants and fungi have various mechanisms to quickly scavenge and degrade it to reduce unnecessary damage (Gil-ad *et al.*, 2000; Neill *et al.*, 2002). These scavenging mechanisms in plants include both enzymatic processes such as catalases or peroxidases, and non-enzymatic antioxidants such as ascorbic acid or tocopherols (Neill *et al.*, 2002; Quan *et al.*, 2008). In fact, the presence of moderate H₂O₂ concentrations in plants can actually provide protection against damage from other ROS by inducing enzymatic antioxidant activity (Gechev *et al.*, 2002).

The total quantity of H₂O₂ in plants, regardless of the mechanism by which it is produced, is limited by several factors. Presence of H₂O₂ in plants is highly localized (Hahlbrock *et al.*, 1995; Bestwick *et al.*, 1997), and relatively short-lived due to the decomposition of H₂O₂ to water and oxygen. H₂O₂ forms, accumulates, acts, and degrades within a matter of minutes to hours (Bolwell and Wojtaszek, 1997; Levine *et al.*, 1994; Neill *et al.*, 2002), while oxalic acid and oxalate salts are more

stable. Plant mechanisms to scavenge and degrade H₂O₂ can be up-regulated in response to exogenously-applied or OxO-generated H₂O₂ (Gechev *et al.*, 2002; Wan *et al.*, 2009).

Production of H₂O₂ by OxO is strictly limited by availability of oxalate as a substrate (Figure 4.1a and Section 6.3.2), which varies widely in concentration between plant sources (McCarroll and Thor, 1978; Haytowitz and Matthews, 1984). In at least one reported instance of a plant using H₂O₂ produced by OxO, the plant also synthesizes the oxalate substrate in situations when H₂O₂ is needed (Davoine *et al.*, 2001). Additionally, the OxO reaction is ultimately self-limiting as it is inhibited by relatively high concentrations of H₂O₂ ($> \sim 10 - 20$ mM) (Cassland *et al.*, 2010; Goodwin *et al.*, 2017). In plants, these inhibitory concentrations of H₂O₂ are unlikely to be produced by OxO outside of highly localized areas in plant cells (Dumas *et al.*, 1995; Bestwick *et al.*, 2001), especially when the OA substrate is limiting, but are of greater interest in potential industrial applications of oxalate oxidase (Cassland *et al.*, 2010).

4.3.4 Indirect effects of OxO and H₂O₂ in plant defenses

Cytosolic Ca²⁺ and H₂O₂ concentrations have been shown to rise together in response to fungal elicitors and pathogen invasion (Hahlbrock *et al.*, 1995; Levine *et al.*, 1996). Ca²⁺ influx helps to sustain the oxidative burst of H₂O₂ needed for cell wall lignification as well as the hypersensitive response (Levine *et al.*, 1996).

Oxalic acid secreted by some fungal pathogens chelates Ca²⁺ ions as calcium oxalate (Lane, 2002). While some plants use calcium oxalate as a means of storing calcium (Section 4.1), in other plants this can inhibit calcium-dependent host plant pathogen responses and weaken cell walls (Bateman and Beer, 1965). Oxalic acid also suppresses the oxidative burst through mechanisms unrelated to its cation binding capacity (Cessna *et al.*, 2000). Degradation of oxalic acid by OxO releases Ca²⁺ (immobilized as calcium oxalate) and produces H₂O₂, both of which can help to re-stabilize cell walls and allow stress- or pathogen-related cell signaling to resume. Model plants transformed with OxO have specifically been shown to have enhanced hydrogen peroxide-based defense responses (Wei *et al.*, 2015).

4.4 Pathogen tolerance provided by endogenous OxO in crops

Germin OxOs in wheat enhance fungal pathogen resistance in part by accumulating in papillae formations. Papillae are appositions formed on the inner surface of plant cell walls where fungal tissue is penetrating (Thordal-Christensen *et al.*, 1997; Wei *et al.*, 1998; Schweizer *et al.*, 1999). Schweizer *et al.* (1999) found that GLPs and germin OxOs accumulate at the site of papillae formations in wheat leaves being attacked by powdery mildew (*Blumeria graminis tritici*). They also found high concentrations of H₂O₂ within the papillae formations, making conditions less suitable for fungal invasion. Transient expression of germin OxOs in wheat leaves reduced fungal penetration by 20 – 65% after exposure to *Blumeria graminis tritici* (Schweizer *et al.*, 1999).

Barley (*Hordeum vulgare*) has also demonstrated resistance to *Blumeria graminis tritici* associated with an OxO isoform. Zhang *et al.* (1995) observed increased expression of OxO in barley tissues, and increased OxO activity increased as a result of elicitors produced by powdery mildew infection. Results from their assays strongly suggest powdery mildew resistance was conferred by the sustained H₂O₂ oxidative burst correlated to OxO concentration and OxO activity spikes, especially in papillae formations (Zhang *et al.*, 1995). Barley leaves showed a sustained increase

in OxO protein and expression in epidermal cells up to 96 hours after *Blumeria graminis tritici* inoculation (Wei *et al.*, 1998). Thordal-Christensen *et al.* (1997) found H₂O₂ production in papillae could be used to prevent further fungal penetration as well as signal neighboring cells of the fungal presence. They also determined H₂O₂ concentrations at different points in time after invasion could account for multiple pathogen responses associated with OxO-generated H₂O₂ (Thordal-Christensen *et al.*, 1997). Constitutive expression of endogenous OxO associated with constitutive defenses against pathogens has been reported in ryegrass, though in this instance OxO is apparently part of a more complex response involving plant-generated oxalate and other compounds, which affect both pathogen responses and senescence (Davoine *et al.*, 2001).

While OxO is most prevalent in true cereal crops (i.e. monocots), similar oxalate-degrading mechanisms may be employed by other crops (dicots) to tolerate oxalic acid-secreting pathogens. Some varieties of tomato (*Lycopersicon esculentum*) show increased oxalate oxidase activity as a response to exogenously applied OA. Tomato plants exposed to low levels of OA tolerated subsequent *Botrytis* infections more effectively than controls treated with distilled water (Sun *et al.*, 2019). White bean (*Phaseolus vulgaris*) is typically susceptible to white mold (*Sclerotinia sclerotiorum*), but one cultivar has been discovered that tolerates *Sclerotinia* infections (Tu and Beversdorf, 1982). This cultivar was observed to be tolerant to oxalic acid (Tu, 1985), and later incorporated into breeding programs to produce additional *Sclerotinia*-tolerant cultivars (Tu, 1989). Tu (1985) did not propose or test for a specific mechanism for degradation of oxalic acid in white bean, but the tolerance mechanism (i.e. a fungus infected the plant, but caused less damage) is characteristic of an oxalate-degrading enzyme (Kumar *et al.*, 2019).

Most of the above examples of endogenous OxO activity involve temporary, localized, or induced OxO expression. This is presumably because overexpressing a defense response typically has metabolic costs (Karasov *et al.*, 2017), resulting in selection pressures for optimal (minimally functional) expression levels. However, to our knowledge constitutive expression of OxO in transgenic plants has not been reported to be detrimental (Section 4.5), so risks of higher expression (apart from possible metabolic costs) are unlikely.

4.5 Use of OxO as a transgene

Oxalate oxidase has been transformed into a variety of plants, most frequently to provide tolerance to fungal pathogens, but with some other documented effects as well. Many of these are described in a recent review by Ilyas *et al.* (2016), and other applications such as medicine and nutrition are described in Section 4.1. The following subsections describe some of these applications in plants other than chestnuts; OxO in Darling 58 specifically is described starting in Section 6.

4.5.1 Tolerance to oxalic acid-secreting fungal pathogens

Numerous studies have shown increased resistance or tolerance to fungal pathogens in transgenic plants expressing germin OxOs or OxO isoforms. *Sclerotinia sclerotiorum*, commonly referred to as white mold, is a multi-host, oxalic acid-secreting fungal pathogen. This fungus is known to be pathogenic to more than 400 plant species, including many valuable crops (Purdy, 1979; Lu, 2003). Many cereal crops tolerate *Sclerotinia* (likely due to their expression of OxO), so a common recommendation is to rotate *Sclerotinia*-susceptible broadleaf crops with OxO-expressing grass

crops (Section 5.3) (Nelson *et al.*, 1989; Peltier *et al.*, 2012). Alternatively, resistance to this pathogen has been demonstrated in several otherwise susceptible plants transformed with OxO isoforms (Donaldson *et al.*, 2001; Lu, 2003; Livingstone *et al.*, 2005; Zhang *et al.*, 2018). For example, post-inoculation survival rates of OxO-expressing transgenic soybeans (100% survival) far exceeded that of the control parental lines (57%) as well as the commercially available “resistant” non-transgenic controls (86%) (Donaldson *et al.*, 2001). Notably, at least one study shows that while transgenic OxO-expressing soy tolerated *Sclerotinia* infection with less damage (smaller lesions), expression of OxO did not inhibit initial infection or lesion formation (Davidson *et al.*, 2016). Additionally, Thompson *et al.* (1995) demonstrated enhanced tolerance to exogenously supplied oxalic acid in transgenic canola that is otherwise susceptible to *Sclerotinia*.

Peanut (*Arachis hypogaea*) is another important crop severely affected by *Sclerotinia minor*, and possibly the most extensively studied crop transformed with OxO (Hu, J. *et al.*, 2015; Balota *et al.*, 2015). Hu *et al.* (2016) found in a three-year study that the OxO transgenic peanut cultivar retained high levels of resistance to the target pathogen, *S. minor*, but had similar levels of resistance as non-transgenic cultivars to other common, non-target peanut pathogens. Peanut quality and composition in these transgenic lines were similar to those in the non-transgenic cultivar (Hu *et al.*, 2014). Livingstone *et al.* (2005) transformed peanut embryos with a barley OxO isoform to confer resistance to *S. minor*. Transgenic peanut lesion size after inoculation was reduced by 75% to 97% compared to non-transgenic controls (Livingstone *et al.*, 2005). They also noted that lesion morphology on transgenic plants mimics that of a hypersensitive response rather than that of pathogenic lesion. Despite the use of the word “resistance” in the publications cited above, it appears that OxO has more accurately resulted in tolerance to this fungal pathogen rather than true resistance, since their goal was to degrade oxalic acid (reducing the *impact* of infection) rather than to kill or deter the pathogen itself (reducing the *incidence* of infection) (Tiffin, 2000; Section 5). Additionally, susceptibility to other fungi in OxO-expressing transgenic lines was unchanged (Hu *et al.*, 2016), reinforcing the lack of broad-spectrum antifungal mechanisms.

Hu *et al.* (2003) transformed a wheat OxO into sunflower (*Helianthus annuus*) to provide resistance to *Sclerotinia sclerotiorum*. Overexpression of OxO in transgenic lines produced significantly enhanced resistance when inoculated with the *Sclerotinia sclerotiorum* compared to control non-transgenic lines. Petioles were inoculated with *Sclerotinia sclerotiorum*, and resulting lesions on the transgenic plants were 6-fold smaller after 10 days than those on non-transformed controls (Hu *et al.*, 2003). Other oilseed crops like canola (Lu, 2003) and rape (Dong *et al.*, 2008) have been transformed with barley OxO to confer resistance to *Sclerotinia sclerotiorum* as well.

Tomato plants are susceptible to two oxalic acid-secreting fungal pathogens, *Botrytis cinerea* and *Sclerotinia sclerotiorum*. At least some varieties of tomato express endogenous OxO genes (Sun *et al.*, 2019), but Walz *et al.* (2008) transformed tomato with a wheat OxO to increase the plant defense response. In *Botrytis cinerea* inoculation experiments, all transgenic events showed increased pathogen resistance compared to wild type plants. The best transgenic tomato lines were also inoculated with *Sclerotinia sclerotiorum*, resulting in greatly reduced lesion size and symptom progression in OxO-expressing tomato lines when compared to wild type plants (Walz *et al.*, 2008).

Hybrid poplar (*Populus x euramericana*) was transformed with a wheat OxO in our lab at ESF to demonstrate OxO's ability to provide resistance to an oxalic acid-secreting fungal pathogen in a woody tree. When oxalic acid tolerance assays were performed on transgenic and non-transgenic leaf discs, transgenic tissue maintained higher percentages of green living leaf area and was able to raise the pH of the surrounding solution (Liang *et al.*, 2001). Under the hypothesis that increasing oxalic acid tolerance can lead to pathogen tolerance, leaf discs were also treated with a *Septoria musiva* conidia suspension. Significantly less necrotic area developed on transgenic leaf discs compared to the non-transgenic controls. Few other woody species have been transformed with OxO, but before testing whole plants, American chestnut callus tissue was also transformed with wheat OxO and subjected to oxalic acid assays (Welch *et al.*, 2007). Lignin content significantly decreased in non-transgenic tissue after exposure to oxalic acid, but lignin was retained in OxO-transformed callus. Maintaining lignin content and increasing oxalic acid tolerance in callus tissue suggested that OxO could play an important role in conferring *Cryphonectria parasitica* resistance to American chestnut trees.

4.5.2 Responses to non-OA-secreting fungi, insects, and abiotic stress

In addition to enhanced tolerance to OA-secreting pathogens, where the OA-degrading mechanism of OxO has a clear role, there are several examples of transgenic OxO-expressing plants that show resistance or tolerance to other stresses. Examples of OxO transgenes being used against other fungal and oomycete pathogens include enhancing resistance to *Rhizoctonia solani* in rice (Molla *et al.*, 2013), to *Phytophthora infestans* and *Streptomyces reticuliscabiei* (but not *Erwinia carotovora*) in potato (Schneider *et al.*, 2002), and to *Phytophthora colocasiae* in taro (He *et al.*, 2013). Along with the OA-specific examples in Section 4.5.1, these are just a few of the many studies that have used transgenic approaches to either introduce an OxO isoform or upregulate an existing OxO to provide fungal defense benefits to a host plant. A recent review by Moosa *et al.* (2017) further describes transgenic applications of GLPs and OxOs for defense against a variety of fungal pathogens.

In certain situations, OxO has also been correlated with reduced herbivory by insects. Ramputh *et al.*, (2002) transformed corn with wheat OxO to reduce herbivory by the European corn borer (*Ostrinia nubilalis*). Tunneling was reduced by up to 50% in transgenic lines, likely due to lignification of cell walls (which can be facilitated by the presence of additional H₂O₂), H₂O₂ signaling predation resistance mechanisms, or H₂O₂'s direct effect on insect physiology (Ramputh *et al.*, 2002). Mao *et al.* (2007) looked at alterations of secondary metabolites due to OxO in transgenic corn and correlations to reduced insect herbivory. While concentrations of ferulic acid were increased in transgenic lines, the authors suggest that reduction of herbivory was more likely due to the toughness of the tissue associated with H₂O₂-induced lignification (Mao *et al.*, 2007). It is important to note that hydrogen peroxide produced in OxO-transformed corn leaves may actually come from an enzymatic process called superoxide dismutase (Ramputh *et al.*, 2002; Woo *et al.*, 2000), which only occurs in neutral or high pH environments (Section 6.3.2).

Apart from pest and pathogen resistance, endogenous oxalate oxidase has been reported to be associated with abiotic stress tolerance in some scenarios, likely due to H₂O₂-induced stimulation of endogenous response pathways, growth reductions, or activation of antioxidants (Lane *et al.*, 1993; Hurkman and Tanaka, 1996; Singh *et al.*, 2006; Dunwell *et al.*, 2008). However, correlations

between abiotic stress and expression of endogenous OxO would not necessarily be applicable to transgenic plants, where OxO expression is not controlled or induced by the same mechanisms. Only a few studies have specifically investigated effects of OxO transgenes on abiotic stress: OxO expression increased tolerance to oxidative stress in transgenic tobacco (Wan *et al.*, 2009), and tomatoes with OxO produced more fruit under salt stress (Dessalegne *et al.*, 1997), but OxO did not consistently affect salinity responses of transgenic potato in tissue culture or greenhouse conditions (Turhan, 2005). More broadly, the hypersensitive response is only one of many factors that contribute to stress tolerance in trees: comprehensive abiotic stress tolerance is a multigenic trait governed by many stimuli that control several mechanisms at different scales (cellular, organ, physiological, morphological, or population) (Sairam and Tyagi, 2004; Harfouche *et al.*, 2014, Polle *et al.*, 2019).

In cases where the stress or pathogen does not employ oxalic acid as a virulence factor, any observed resistance correlated with OxO expression is likely due directly or indirectly to production of H₂O₂ (Sections 4.3 and 6.3). As described previously, effects of H₂O₂ (both directly and due to resulting lignification) would be limited spatially and temporally by the presence of the oxalic acid substrate, without which H₂O₂ would not be produced (Section 4.3.3).

5.0 Tolerance as a form of plant defense against pathogens

5.1 Terminology of pathogen tolerance and resistance

Given the mechanism of oxalate oxidase as described above (Section 4.4), plants that employ this plant defense mechanism in response to a fungal pathogen would most appropriately be considered “tolerant” to that pathogen. There are varying definitions of tolerance within plant pathology literature, but as we use it here, tolerance is a type of plant defense in which the host maintains its fitness or yield despite damage caused by the pathogen, whereas resistance is a type of defense in which the incidence of infection or performance (survival) of the pathogen is reduced (Schafer, 1971; Rosenthal and Kotanen, 1994; Rausher, 2001). Apart from tolerance, resistance can be further characterized as either causing *avoidance* by pests (changing a pest’s preference or behavior) or *antibiosis* (reducing performance of the pest, typically by generation of toxic pesticidal compounds) (Tiffin 2000).

Since these various mechanisms are not mutually exclusive, it may be helpful to visualize plant defense responses on a spectrum from resistance to tolerance. At the resistance end of the spectrum, plant traits may kill the pest or prevent it from reproducing, while at the tolerance end, plant traits may allow the plant to simply tolerate the continuing presence of the pest without necessarily affecting the pest (Woodcock *et al.*, 2018). Tolerance and resistance mechanisms often coexist in plants (Pagan and Garcia-Arenal, 2018), so it is possible to stack tolerance and resistance mechanisms together.

Tolerance, which does not involve changing preference or performance of the pest, is often accomplished by mechanisms of compensatory growth and/or the production of detoxifying compounds (Strauss and Agrawal, 1999; Tiffin, 2000; Woodcock *et al.*, 2018), both of which allow the pest to survive and reproduce on the host. Compensatory growth is more commonly described in the context of herbivorous insect pests, but detoxification of fungal toxins in plant-microbe interactions is well documented as well (Karlovsky, 1999; Völkl *et al.*, 2004; Khallaf, 2013; Mandalà *et al.*, 2019). American chestnut does exhibit compensatory growth in response to infection by *Cryphonectria parasitica*, but this growth is insufficient to prevent severe damage to the host tree (Shigo, 1982).

Because a primary mechanism of oxalate oxidase in fungal disease response is to detoxify oxalic acid, rather than to kill a pest or to prevent infections, in this petition we prefer to use the term “tolerance” to describe this particular mechanism of plant defense. However, more general considerations of pathogen interactions with plants (e.g. chestnut blight as described in Section 3) may still be discussed in terms of “resistance,” because the quantitative resistance seen in Asian species of chestnut may incorporate multiple mechanisms. Additionally, essentially all chestnut blight literature (e.g. van Fleet, 1914; Graves, 1950; Dietz, 1978; Clark *et al.*, 2019) and some pathogen tolerance literature (Schafer, 1971; Miller *et al.*, 2005; Woodcock *et al.*, 2018) use the term resistance in a general sense to potentially incorporate tolerance mechanisms.

5.2 Necrotrophic and saprophytic life styles of *Cryphonectria parasitica*

In order to properly understand the sustainability of the OxO disease tolerance mechanism, it is important to distinguish necrotrophic, saprophytic, opportunistic pathogens such as the chestnut

blight fungus from those which are obligate parasites. Pathogenic fungi such as *Sclerotinia* spp. and *Cryphonectria parasitica* are termed “necrotrophic” (from the Greek words for ‘dead’ and ‘nourishment’) pathogens, meaning that they kill their host plant cells in advance of invasion, subsisting only on dead host tissues (such as dead bark and vascular cambium in chestnut) rather than actively consuming live tissue. Necrotrophic fungi are mainly saprophytes, meaning they are fully capable of deriving nutrition from decaying plant residues, such as may be found on soil surfaces in agricultural settings or in the detritus (duff) and subtending shallow layers of forest soils. (The term *saprotrophic* is used interchangeably in some contexts: this refers to subsisting on any dead or decaying organic matter, while *saprophytic* more specifically indicates dead material from plants.) In contrast, biotrophic fungi require living host tissue to survive. These categories are not phylogenetic groupings: they are based on lifestyle or behaviors of pathogens, so there are overlapping or intermediate categories and they may change over time.

In the case of chestnut blight, the fungus initially infects a tree by entering and colonizing wounds in the bark where it absorbs nutrients from dead or dying tissue. The fungus also releases oxalic acid, which damages living tissue on susceptible American chestnuts, killing bark, cambium, and xylem tissues of the tree stems and releasing additional plant cell contents, which the fungus can use in its own metabolism. This damage results in areas of dead cells known as cankers. Where the innermost bark layer (phloem) is damaged or killed, it can no longer transport sugars from the leaves to the tree roots. If this damage encircles (girdles) the tree, the portion of the tree above the infection soon dies.

While the released oxalic acid damages plant cells, the fungus is not dependent on this mechanism to thrive. Even when it cannot produce any oxalic acid, *C. parasitica* invades wounds, colonizes tissue, and replicates on American chestnuts (Chen *et al.*, 2010). Indeed, in its natural range, *C. parasitica* colonizes the more resistant Chinese chestnut upon which it thrives as a saprophyte and weak parasite (Clapper, 1952), and typically produces only superficial cankers that generally do not kill the tree (Hebard *et al.*, 1984). In North America, the fungus similarly survives on several endemic trees such as oaks (*Quercus* spp.), hickory (*Carya* spp.), sumac (*Rhus* spp.), maples (*Acer* spp.), and others, all of which are less susceptible to oxalic acid damage than the American chestnut (Stipes *et al.*, 1978; Nash and Stambaugh, 1987; Baird, 1991; Davis *et al.*, 1997; Rigling and Prospero, 2018). When the blight fungus is interacting with these less susceptible trees, it would generally not be considered pathogenic or necrotrophic, since it does not actively kill tissue, but it is still saprophytic. The fungus has also been reported to survive even more abundantly on dead American chestnut stems as a saprophyte than on a live American chestnut as a pathogen (Prospero *et al.*, 2006). Perpetual colonization of these other forest species by *C. parasitica* is a principal reason that the fungus (and resulting chestnut blight) remains prevalent in North America, even as the American chestnut itself has nearly disappeared as a canopy tree species from eastern woodlands and is considered functionally extinct.

5.3 Stability of fungal pathogen tolerance conferred by OxO

One important consideration for restoration of a wild organism is to employ a durable mechanism of plant defense. Some host/pathogen models predict that tolerance mechanisms are especially advantageous for plants with slower growth and longer lives (Pagan and Garcia-Arenal, 2018), so such mechanisms may be particularly well-suited for trees. Tolerance has also been suggested to

be a more evolutionarily stable form of defense than other forms of resistance since it increases host fitness without directly affecting the pathogen community (Tiffin, 2000; Ayres and Schneider, 2012), avoiding the “arms race” of specific resistance genes or mechanisms between pest and host (Rosenthal and Kotanen, 1994; Strauss and Agrawal, 1999; Roy and Kirchner, 2000). The stability of tolerance mechanisms has been proposed as a means of precluding counter-resistance management plans (Rausher, 2001), an important consideration for wild trees. In the case of truly resistant plants that produce toxins to kill or deter pests, a “high dose/refuge” strategy is often proposed to prevent pathogen evolution, where susceptible (non-toxic) hosts are planted along with their resistant counterparts, to reduce the overall selective pressure on pests or pathogens (Caprio and Sumerford, 2007; Gryspeirt and Grégoire, 2012; Gould *et al.*, 2018). In the case of a tolerant host without any toxicity mechanism, all hosts essentially function as refuges, which should allow for a very stable relationship with the pathogen, making novel plant pest risks unlikely. Therefore, tolerance appears to be an ideal mechanism to durably enhance plant defenses to pathogens without novel plant pest risks.

Oxalate (oxalic acid) has long been well known as a general toxin in mammalian biochemistry (Christison and Coindet, 1823; Brown and Gettler, 1922; Hodgkinson, 1977; Svedružić *et al.*, 2005), as well as in plants (Sections 3.2 and 4.3). In chestnut tree tissues, oxalate can lower the pH at the margin of the chestnut blight canker from a normal pH level of approximately 5.5, to a toxic level of pH 2.8 (McCarroll and Thor, 1978). Oxalate also chelates calcium needed by plants for the formation of pectin (one of the principal substances that binds plant cells together), and suppresses the oxidative burst used by the plant in defense against invading microorganisms (Cessna *et al.*, 2000, Section 4.3.4). In some plant systems oxalate has been shown to induce programmed cell death known as apoptosis (Kim *et al.*, 2008), which can defend a host against biotrophic pathogens, but actually favors necrotrophic pathogens (Sections 4.3 and 6.3.2). In many host plants, naturally occurring oxalate oxidase (OxO) functions to degrade and remove oxalate so it can no longer exert adverse effects.

For another example of the evolutionary stability of oxalate oxidase as a disease tolerance mechanism, it is useful to consider fungal pathogens such as *Sclerotinia* spp., with an oxalic acid virulence mechanism similar to that exhibited by the chestnut blight fungus (Bateman and Beer, 1965; Noyes and Hancock, 1981). These and similar fungal pathogens have been extensively studied in agricultural systems because they are highly destructive to crop yields, and are problematic due to recalcitrance in the face of typical control measures (Steadman, 1979). As these soilborne fungal pathogens infect their host plants, they secrete oxalic acid. This acid kills the cells of their prospective host plants, so that the mycelium (the vegetative body of the fungus) can then grow and spread within the killed plant cells, absorbing nutrients from the destroyed host tissues.

Grasses, and even several species of dicotyledonous plants, apparently employ OxO as a tolerance mechanism against oxalic acid-producing necrotrophic fungi (Section 4). We do not know of any grass species that are susceptible to necrotrophic organisms that secrete oxalic acid as their virulence mechanism. In agriculture, in order to take advantage of the disease suppressive effect from the oxalate detoxification mechanism in grasses, it is routinely recommended to rotate *Sclerotinia*-susceptible broadleaf crops such as soybean (Peltier *et al.*, 2012), sunflower (Nelson *et al.*, 1989), and canola (Canola Council of Canada, 2017) with *Sclerotinia*-tolerant grass crops (corn,

wheat, barley, rye, etc.). This minimizes survival and proliferation of necrotrophic *Sclerotinia* spp. Despite this common practice, and despite the co-occurrence of *Sclerotinia* with OxO-producing monocotyledonous crops for millennia, *Sclerotinia* has apparently not evolved any mechanism to overcome the oxalate tolerance mechanisms in these species. OxO-based tolerance to other fungal pathogens with similar virulence mechanisms should be similarly stable and therefore not increase related plant pest risks.

Several types of plant pathogens, as well as most animal and human pathogens (particularly viruses) are biotrophic, meaning that they require living host tissue to invade and replicate. Examples of plant pathogens that are obligate biotrophs are those which cause "rusts" (wheat leaf rust, stem rust, corn rust, etc.), powdery mildews, and downy mildews. With obligate biotrophic parasites, there is extreme selection pressure for the pathogen to maintain virulence. If a gene for resistance exists in the host, by definition there is a reciprocal gene for avirulence (inability to cause disease) in the pathogen (Brading *et al.*, 2002). This is known as the gene-for-gene relationship of plant disease, which was well documented more than 75 years ago (Flor, 1942). Thus, throughout the history of crop breeding for resistance to rust diseases, every time a resistance gene is found and bred into the wheat population, selection pressure is immediately placed on the rust pathogen's population to increase the frequency of an allele for virulence to circumvent the host resistance gene. These same principles also pertain to resistance breeding to control powdery mildews and downy mildew pathogens which are caused by obligate parasites, and where gene-for-gene relationships are well documented (Huang *et al.*, 1997; Liu *et al.*, 2017). As a result, genetic resistance to obligate biotrophic parasites is an ephemeral solution, which has frustrated plant breeders throughout the history of modern agriculture. Genetic resistance to obligate pathogens in improved crop varieties typically remains stable for a few years until new, virulent pathogen varieties develop which circumvent the resistance mechanism, which then prompts the discovery and incorporation of additional resistance genes.

In contrast, since non-obligate, principally necrotrophic pathogens like the chestnut blight fungus can and do live and reproduce as saprophytes, there is minimal selective pressure on them to devise alternative virulence mechanisms because they do not require living hosts. This has been demonstrated in Asia where *C. parasitica* has not overcome the tolerance mechanisms found in Asian chestnuts and still lives principally as a saprophyte or weak pathogen on those species. As has been discussed above (Sections 3 and 5.2), long after most American chestnuts and chinquapins died from the initial blight progression wave, the blight fungus continues to live ubiquitously in the forest litter and on tree surfaces (such as oaks) as a saprophyte, as well as in parasitic form on chestnut stump sprouts.

5.4 Endogenous oxalic acid tolerance traits in *Castanea*

It is generally understood that the blight tolerance observed in Chinese chestnut (and other *Castanea*, Section 3.1) is due to multiple genes working together (Kubisiak *et al.*, 1997; Steiner *et al.*, 2017; Westbrook *et al.*, 2019b). In fact, several "cisgenes" (genes from the related Chinese chestnut) have been identified (Barakat *et al.*, 2012) and transformed into American chestnut by our lab at ESF. Preliminary results indicated that individual cisgenes provided, at best, only moderately enhanced blight resistance (Nelson *et al.*, 2014), though there is potential that stacking multiple cisgenes or enhancing expression of these genes may offer meaningfully

increased blight resistance. Relative blight resistance as reported in Chinese chestnut and Allegheny chinquapin (Graves, 1950) is apparently due *in part* to OA tolerance in these species. Preliminary leaf disc assays (not shown) on putatively blight-tolerant Ozark chinquapin (Bost, 2019) have shown similar tolerance to oxalic acid. There are likely other tolerance or resistance mechanisms involved as well, but differential tolerance to oxalic acid has been demonstrated by soaking leaf discs in oxalic acid and observing how much of the tissue becomes necrotic (Figure 5.4a; Section 6.3.1). These assays included 1.5 cm diameter leaf discs, soaked overnight in 50mM oxalic acid, then scanned to calculate remaining green/healthy leaf area relative to total disc area.

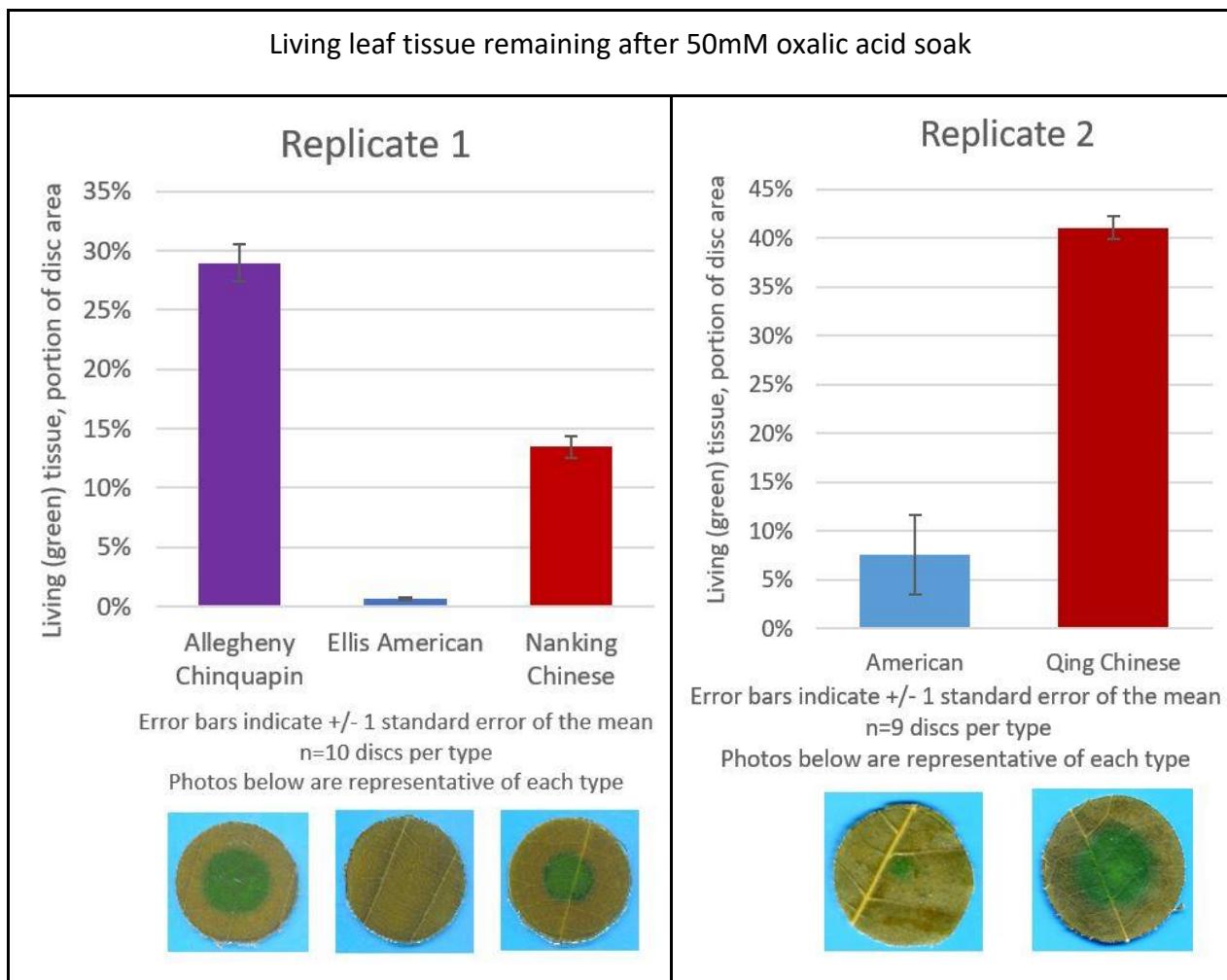


Figure 5.4a. Results from two oxalic acid soak assays of chestnut leaf discs, showing higher tolerance to oxalic acid in Chinese chestnut and Allegheny chinquapin compared to American chestnut. Data are not necessarily comparable between different replicates. Some data from Replicate 2 are also shown as part of Figure 6.3.1a.

Since neither Chinese chestnut nor chinquapins show oxalate oxidase activity, and genomic DNA sequence searches have not found a gene that matches more than 79% identity to OxO (Section 5.5), these species must have a separate mechanism for tolerating or degrading oxalic acid. Research is underway to elucidate oxalic acid tolerance mechanisms in both Chinese chestnut and chinquapins; any details relevant to this petition will be published and/or shared as they become available.

Besides oxalate oxidase, there are two other known pathways by which plants can potentially metabolize oxalic acid (Kumar *et al.*, 2019). One uses oxalate decarboxylase (Section 4.1), but we have not identified a gene in Chinese chestnut encoding this enzyme. The other possibility is a four-enzyme pathway first described in *Arabidopsis* (Figure 5.4b) (Foster *et al.*, 2012). With currently available genome data, three of the four enzymes needed for this pathway have been identified in Chinese chestnut. Only the Formyl-CoA hydrolase (or an equivalent enzyme, marked with a "?" in Figure 5.4b) still needs to be found to support this hypothesis. Regardless of the

specific mechanism in Chinese chestnut or chinquapins, the oxalic acid tolerance *trait* is not new to chestnut species and therefore enhancing it in the American chestnut should not introduce novel plant pest risks.

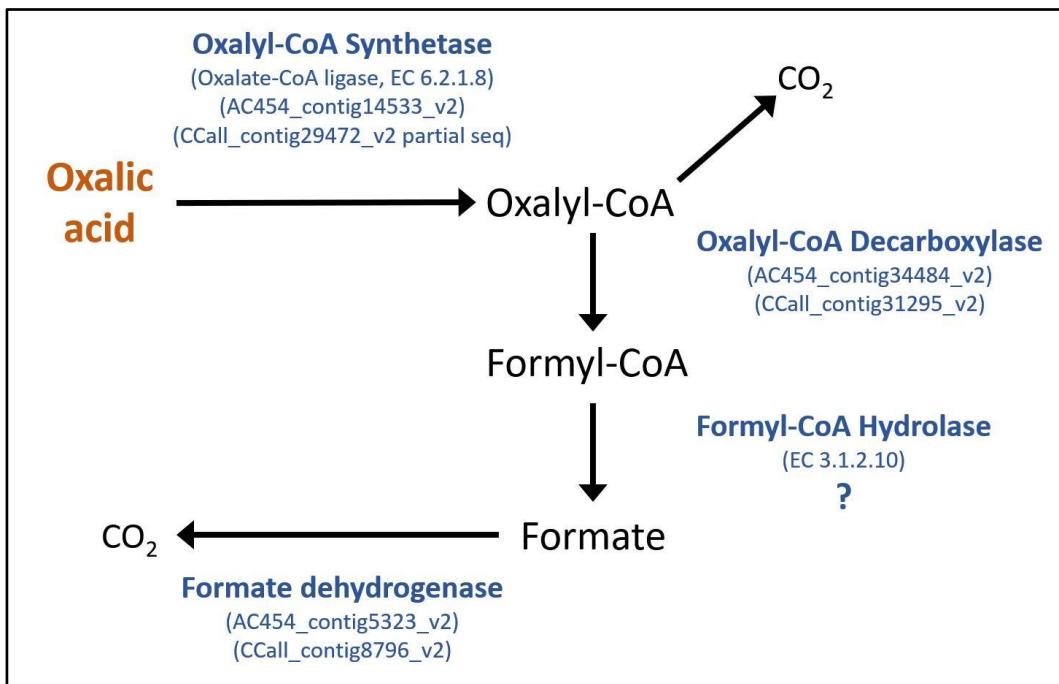


Figure 5.4b. Enzymatic pathway used by Arabidopsis (and hypothetically Chinese chestnut) to help tolerate oxalic acid. Chestnut transcriptome contig numbers included (based on the Chinese chestnut genome Transcriptome Version 081508, accessible via hardwoodgenomics.org; Staton et al., 2015).

5.5 Endogenous *Castanea* genes similar to OxO

While a gene for OxO specifically has not been reported in any non-transgenic *Castanea*, several similar genes are present in chestnut. Oxalate oxidase as encoded by wheat and other cereal grains belongs to the family of plant proteins called germins (Pan *et al.*, 2007; Section 4.1), and there have been multiple germins identified in various species of *Castanea*. The following alignments between native chestnut genes and wheat OxO (Figure 5.5a) are reported in terms of identity (proportion of exact amino acid matches between two sequences) and similarity (proportion of matches including chemically or functionally similar amino acids, called “Positives” in NCBI results). All gene references described in this section are available at hardwoodgenomics.org unless otherwise noted.

C. mollissima has a sequenced germin that shares 50% amino acid identity and 65% similarity with wheat OxO, while a separate gene from *C. dentata* (full sequence from ESF unpublished; see AC454_contig659_v3 via hardwoodgenomics.org for partial sequence) also shares 50% amino acid identity and 66% similarity with wheat OxO (GenBank: M21962.1). This same gene from *C. dentata* shares 53% identity and 68% similarity with oxalate oxidase from barley (GenBank: Y14203.1; alignment not shown). Like OxO, these proteins all have conserved germin motifs (shared protein structures typical of germins including OxO, noted in Figure 5.5a). These include

an active site histidine cluster with a metal ion-binding region, and their overall protein structures are the characteristic germin β -barrel (Gane *et al.*, 1998), which indicates similar function and possibly shared evolutionary relationships between proteins. While these germins in Chinese and American chestnuts have not been shown to have oxalate oxidase activity, they are related structurally.

| Range 1: 4 to 214 Graphics Castanea mollissima germin alignment | | | | | |
|---|---|---|--------------|--------------|------------|
| Score | Expect | Method | Identities | Positives | Gaps |
| 191 bits(486) | 2e-66 | Compositional matrix adjust. | 108/218(50%) | 143/218(65%) | 11/218(5%) |
| Query 8 | VAGLFAM--- | LLLAPAVLATDPDPLQDFCVADLDGKAVSVNCHTCKPMSE-AGDDFLFSS | 63 | | |
| Sbjct 4 | + LF M ++L PA A DPD LQD CVADL V VNG TCK ++ + DF F | ILALFVMTFAVVVLGPA--AADPDLLQDVCAVLTS-GVKVNGFTCKDIANISAADFSFDG | 60 | | |
| Query 64 | KLAKAGNTSTPNCSAVTELDVAEWPGLTGVSMRNFDFAPGGTNPHIHPRATEIGIVM | strand A strand B strand C strand D | 123 | | |
| Sbjct 61 | LAK G T+ GS VT +V + PG NTLGVS++R+D+APGG NPPH HPRATE+ V+ | -LAKPGLTNNTFGSLVTAANVQKIPGLNLGVSLSRIDYAPGGLNPHTHPRATEMVFVL | 119 | | |
| Query 124 | KGELLVGILGSLSGNKLYSRVVRAGETFLIPRGLMHFQFNVGKTEASMVVSFNSQNPPI | strand E strand F strand G strand H strand I | 183 | | |
| Sbjct 120 | +GEL VG + + N L S+ ++ GE F+ P+GL+HFQ N GK A+++ +FNSQ PG | EGELNVGF---ITTANVLISKSIIKKGEIFVFPKGVLVFQKNNGKVPAAVIAAFNSQLPGT | 176 | | |
| Query 184 | VVFVPLTLFGSNPPPIPTPVLTAKLRVEARVVELLKSKFA 221 | strand J | | | |
| Sbjct 177 | + TLF + PP+P VLTKA +V + VE +KS+FA | QSIATTLFAATPPVPDNVLTKAFQVGTKEVEKIKSRFA 214 | | | |

| Range 1: 5 to 205 Graphics Castanea dentata germin alignment | | | | | |
|--|--|---|--------------|--------------|-----------|
| Score | Expect | Method | Identities | Positives | Gaps |
| 190 bits(482) | 6e-66 | Compositional matrix adjust. | 105/208(50%) | 139/208(66%) | 8/208(3%) |
| Query 15 | LLLAPAVLATDPDPLQDFCVADLDGKAVSVNCHTCKPMSE-AGDDFLFSSKLA | strand A | 73 | | |
| Sbjct 5 | KAAGNTST+L PA A DPD LQD CVADL V VNG TCK ++ + DF F LAK G T+ | VVLGPA--AADPDLLQDVCAVLTS-GVKVNGFTCKDIANISAADFSFDG-LAKPGLTN | 60 | | |
| Query 74 | PNGSAVTELDVAEWPGLTGVSMRNFDFAPGGTNPHIHPRATEIGIVMKGELLVGIL | strand B strand C strand D strand E | 133 | | |
| Sbjct 61 | GS VT +V + PG NTLGVS++R+D+APGG NPPH HPRATE+ V++GEL VG | TFGSLVTAANVQKIPGLNLGVSLSRIDYAPGGLNPHTHPRATEMVFLLEGELNVGF-- | 118 | | |
| Query 134 | SLDSGNKLYSRVVRAGETFLIPRGLMHFQFNVGKTEASMVVSFNSQNPPIVVFPLTLFGS | strand F strand G strand H strand I strand J | 193 | | |
| Sbjct 119 | + + N L S+ ++ GE F+ P+GL+HFQ N GK A+++ +FNSQ PG + TLF + | -ITTANVLISKSIIKKGEIFVFPKGVLVFQKNNGKVPAAVIAAFNSQLPGTQSIATTLFAA | 177 | | |
| Query 194 | NPPPIPTPVLTAKLRVEARVVELLKSKFA 221 | | | | |
| Sbjct 178 | PP+P VLTKA +V + VE +KS+FA | TPPVVPDNVLTKAFQVGTKEVEKIKSRFA 205 | | | |

Figure 5.5a. NCBI Protein alignments of *Triticum aestivum* oxalate oxidase (GenBank: M21962.1, labeled “Query”) and sequenced germins from *C. mollissima* (top) and *C. dentata* (bottom), both labeled “Sbjct”, both from hardwoodgenomics.org. Red bars beneath alignments identify conserved active site histidine clusters and blue arrows above alignments identify β -strands (Gane *et al.*, 1998), both of which are characteristic of germin structural motifs.

Chinese chestnut also has a germin-like protein (GLP) that shares a 79% amino acid sequence identity with a putative oxalate oxidase in peanut (Figure 5.5b). Thus it is possible that Chinese chestnuts once had this function in their evolutionary history, or that they have a similar function via a different enzymatic pathway (Section 5.4).

| Sequence ID: Query_162833 Length: 219 Number of Matches: 1 | | | |
|--|--|---|-----------|
| Range 1: 1 to 219 Graphics | | ▼ Next Match ▲ Previous Match | |
| NW Score | Identities | Positives | Gaps |
| 866 | 172/219(79%) | 194/219(88%) | 2/219(0%) |
| Query 1 | MAAILALFVMTFAVVLGP-AAAADPDLLQDVCVADLTSGVKVNGFTCKDIANISAADFSFD | 59 | |
| Sbjct 1 | MKFIGSVLVVTFALVLASTSASDPDALQDLCVADTKSGVKVNGFTCKDAAKVNASDFSSN | 60 | |
| Query 60 | GLAKPGLT-NNTFGSLVTAANVQKIPGLNLTGVSLSRIDYAPGGLNPPHTHPRATEMVFF | 118 | |
| Sbjct 61 | LAKPG+ N TFGS+VT ANV+KIPGLNLTGVSL+RIDYAPGGLNPPHTHPRATE+VFV | 120 | |
| Query 119 | LEGELNVGFITTANVLISKSIIKKGEIFVFPKGVLVHFQKNNGKVPAAVIAAFNSQLPGTQS | 178 | |
| Sbjct 121 | LEG+L+VGFITT+NVLISK+I KGEIFVFPKGVLVHFQKNN PAAVI+AFNSQLPGTQS | 180 | |
| Query 179 | IATTIILFAATPPVPDNVLTKAFQVGTKEVEKIKSRFAPKK | 217 | |
| Sbjct 181 | I TLFAATPPVPDNVLTKAFQVGTKE+EKIKSR APKK | 219 | |

Figure 5.5b. Blastx amino acid alignment of Chinese chestnut germin-like gene “CCall_contig44244_v2” (Query) showing a 79% ID match with a putative oxalate oxidase in peanut (GenBank accession #ABS86851) (Sbjct).

In addition to Chinese chestnut, a GLP has been reported in the European-Japanese hybrid chestnut ‘Bouche de Bétizac’, likely originating from the Japanese chestnut parent (Dini *et al.*, 2012). This hybrid is considered resistant to the chestnut gall wasp, apparently due to a hypersensitive response resulting in hydrogen peroxide production in developing buds. These authors found substantially enhanced expression of a GLP gene correlated with high H₂O₂ concentrations in infested buds of the resistant cultivar, but not in a susceptible control or uninfested buds, suggesting that this chestnut GLP gene may have OxO activity (Dini *et al.*, 2012). However, GLPs are a diverse group of proteins (Ilyas *et al.*, 2016), so it is possible that the H₂O₂ in these chestnut buds is not a direct result of OxO activity, it is not clear where the oxalic acid substrate needed for this reaction would originate. A GLP in European chestnut has also been reported to be upregulated in response to *C. parasitica* inoculation, but it was not further characterized for possible OxO activity (Schafleitner and Wilhelm, 2002).

None of the GLPs described in this section are exactly equivalent to wheat oxalate oxidase, but they do represent related proteins already found in sexually compatible *Castanea* species, which could be integrated into American chestnut genomes via natural or controlled hybridization. Regardless of the specific mechanism, the observed OA tolerance (Section 5.4) and the presence of similar proteins in related species demonstrates that OxO would not impart an entirely new trait to *Castanea*, but rather enhance tolerance already present in this genus to a higher level. Because oxalic acid tolerance is not a new trait to *Castanea*, it is unlikely to produce a novel environmental or plant pest risk.

6.0 Darling 58 transformation and background

Previous sections have described the rationale for submitting this request to USDA APHIS for a determination of nonregulated status for blight-tolerant Darling 58 American chestnut, and described relevant background information about chestnuts, blight, and the OxO enzyme. This section relates these topics to transgenic chestnuts and introduces Darling 58 specifically.

To increase American chestnut's tolerance to chestnut blight, an oxalate oxidase (OxO) gene has been inserted into an American chestnut line known as Ellis. OxO (Section 4), known to detoxify the oxalic acid produced by the fungus, is common in cereal grains and other foods that are eaten regularly by people and livestock. The transgenic American chestnut event described in this petition, known as Darling 58, was transformed using an *Agrobacterium tumefaciens*-based protocol adapted by researchers at ESF (Merkle *et al.*, 1991; Carraway and Merkle, 1997; Xing *et al.*, 1999; Polin *et al.*, 2006). This has resulted in a tolerance mechanism that is inherited and expressed by offspring of Darling 58, and should remain uniquely stable without imposing a selection pressure on the blight fungus.

6.1 Plant material

6.1.1 Ellis recipient line

The isogenic line (recipient/background genome) of Darling 58 is known as Ellis. Ellis was established in tissue culture from a single immature zygotic embryo extracted from wild seed of an American chestnut tree known as Pond #1. This tree is located near Windsor, NY, on the property of a TACF member who reports that in the early 1960s, the property contained hundreds of naturalized American chestnuts, including the Pond #1 tree. To our knowledge, there were no chestnuts from outside sources or other locations planted on this property before the establishment of Pond #1. Presently, most of the trees have either died or been reduced to stump sprouts.

The Ellis genome has recently been sequenced by the Joint Genome Institute (JGI), in collaboration with TACF and our labs at ESF; preliminary results are being applied to ongoing chestnut research (Tuskan *et al.*, 2018; Westbrook *et al.*, 2019b; Section 7.2), and we anticipate a full report will be published soon.

6.1.2 Somatic embryo culture

Ellis somatic embryos were established and maintained in tissue culture following the procedure described by Maynard *et al.* (2015). Briefly, immature burs were collected from the Pond #1 tree approximately 1 month post-anthesis. Nuts were removed from burs, sterilized, and rinsed in sterile distilled water. Nuts were cut open and individual zygotic embryos were transferred to Petri plates containing E1 medium (Appendix II). When the single zygotic embryo for the Ellis line began to multiply, the line was subcultured and transferred to fresh E1 medium every two to three weeks. Media for American chestnut embryo and shoot cultures (Appendix II) were developed over many years by multiple working groups (Merkle *et al.*, 1991; Carraway and Merkle, 1997; Xing *et al.*, 1999; Polin *et al.*, 2006). All development and production of chestnut recipient lines and *Agrobacterium tumefaciens* vectors has taken place in Syracuse, NY, USA.

6.2 Transformation and regeneration processes

6.2.1 *Agrobacterium tumefaciens*-mediated transformation

Ellis somatic embryo clumps were transformed using *Agrobacterium tumefaciens* strain AGL1 containing the construct p35S-OxO (Section 7.1) following the procedure described by Maynard *et al.* (2015). The transformation that ultimately yielded the Darling 58 event was initiated on July 12, 2012. Selected details of this procedure are also included in Appendix II. Briefly, *Agrobacterium tumefaciens* was grown in Luria-Bertani broth, pelleted, resuspended in Virulence Induction medium, and incubated on an orbital shaker for 2 hours. Ellis somatic embryo clumps were mixed for 1 hour with the *Agrobacterium tumefaciens* inoculum, then transferred to a desiccation plate (Figure 6.2.1a, far left), comprised of a sterile 60 x 15 mm Petri dish containing a 55mm Whatman® filter paper slightly moistened with sterile distilled water (Cheng *et al.*, 2003).

Embryos were co-cultivated on desiccation plates with the residual *Agrobacterium tumefaciens* inoculum in the dark for 48 h. Clumps were then transferred to a semi-solid “Agro Kill” medium (containing antibiotics to remove *Agrobacterium tumefaciens*) for 1 week, and then moved to RITA® bioreactors (Figure 6.2.1a, center). Bioreactors were intermittently flooded with a liquid selection medium for four weeks, after which dead tissue was discarded, and any pieces of tissue that remained light in color were transferred to semi-solid E1 selection medium.



Figure 6.2.1a. Left: somatic embryos co-cultivating with *Agrobacterium tumefaciens* on a desiccation plate; middle: somatic embryos in a RITA® bioreactor; right: shoot growing from transformed embryo tissue.

Each clump of surviving (light-colored) tissue was considered a separate event, presumably originating from a single transformed cell (Section 6.2.2). Putative transgenic events were multiplied as somatic embryos for at least one month and then tested for the presence of OxO via polymerase chain reaction (PCR). The PCR that confirmed Darling 58 as a transformation event containing the OxO gene was conducted on November 20, 2012 (Section 7.2.1). Embryos were propagated and monitored for any *Agrobacterium tumefaciens* growth for more than six months before regeneration. Darling 58 embryos have been maintained in tissue culture for more than seven years, during which time no *Agrobacterium tumefaciens* has been observed, even when cultured on a medium without antibiotics.

6.2.2 Regenerating embryos to shoots

Once the presence of OxO in the somatic embryos was confirmed, they were multiplied and regenerated (Figure 6.2.1a, far right) as described by Maynard *et al.* (2015). In previous studies, some of the light-colored sectors on the embryos had originated from multiple independent transformation events, which in turn led to mixed shoot cultures. To avoid this problem, a single shoot was selected from the regenerated transformed material. This individual shoot was multiplied and all resulting propagated material comprising the Darling 58 event is from this single shoot. After the initial PCR to identify putative transformants (Figure 7.2.1a), all tissue used for molecular analyses and other experiments was also derived from this single shoot culture.

6.2.3 Rooting and acclimatization

Darling 58 was rooted via an *ex vitro* rooting procedure, detailed in Oakes *et al.* (2016a). Large shoots were excised from basal callus, dipped in Clonex® rooting hormone gel, and inserted into moistened Jiffy-7® peat pellets. The pellets were enclosed in a clear plastic shoebox, then placed on a light bench for three to four weeks. Once roots emerged from the pellet, the whole pellet was planted in a Stuewe & Sons, Inc. D27L “Deepot” containing Sun Gro® Horticulture Propagation Mix and placed in a high humidity growth chamber for acclimatization (16h photoperiod, 80% relative humidity). After 3 to 6 months, plants were transferred to a greenhouse and repotted if necessary. Once large enough, plants were used for experiments (e.g. Sections 8 and 9) and/or planted in permitted field plots (Appendix I).

6.3 Introduction to blight tolerance in Darling 58

The Darling 58 American chestnut expresses OxO, which detoxifies oxalic acid (OA) by degrading it into carbon dioxide and hydrogen peroxide, effectively neutralizing this acid and minimizing the physical damage to the tree that would otherwise occur. Since OxO has no direct fungicidal mechanism, the fungus and the tree can coexist, just as the fungus coexists with the naturally blight-tolerant Chinese chestnut trees in its native range. One of the byproducts of OxO activity is hydrogen peroxide (H_2O_2), which can have antimicrobial activity if it is present in adequate concentrations. However, as described in Sections 4.3 and 6.3.2, overall H_2O_2 concentrations are unlikely to be substantially increased in Darling 58 chestnuts compared to non-transgenic relatives, because non-transgenic chestnuts also produce H_2O_2 in response to cankers, and OxO production of H_2O_2 is strictly limited by available quantities of oxalic acid.

It is not our intent that the OxO enzyme itself should kill or otherwise directly affect the fungus. Instead, the effect is to neutralize the oxalic acid that has been released by the fungus and prevent the acid from damaging the tree’s tissues and eventually killing the tree. Previous work on the blight fungus has shown that oxalic acid is correlated with total tissue damage resulting from infections, but not necessarily correlated with the ability of the fungus to initiate infections (Section 3.2; Vannini *et al.*, 1993), suggesting that degradation of OA should not inhibit infection by *C. parasitica*. This has also been demonstrated by inoculating susceptible stems with strains of *C. parasitica* that don’t produce oxalic acid: infections are initiated, but damage is substantially limited (Chen *et al.*, 2010). The technology achieves its purpose — preventing the death of the tree that results from oxalic acid damage to the tree’s phloem (or conductive tissues) — regardless of the continuing presence of the fungus and its production of OA.

The Darling 58 tree does not need to resist ongoing colonization, reproduction, or OA production by the fungus to tolerate chestnut blight. This is because the OxO degrades the OA as it is being secreted and prevents it from building up to toxic levels. *C. parasitica* survives on Darling 58 trees much as it does on Asian chestnuts and other tree species. In fact, Darling 58 remains a suitable food source for the fungus while the tree is alive, and any saprophytic activity of the fungus on dead chestnut tissues would not be affected, since oxalate oxidase production can only occur in living tissues and enzymatic activity stops once tissues dry out (Section 9.4).

The blight fungus still colonizes and reproduces on Darling 58 trees, principally in saprophytic rather than necrotrophic form (Section 5.2). We have repeatedly observed that Darling 58 trees can be infected by *C. parasitica* (e.g. Figures 8.1.3a and b) though overall canker size is dramatically reduced compared to susceptible control trees. Continuing survival of *C. parasitica* in both Chinese and OxO-expressing American chestnut stems has been confirmed by re-isolation of the fungus following blight inoculations (Figure 10.5.1c). Identification of the blight fungus following re-isolation was confirmed by morphological observations, and by inoculations with the re-isolated fungus on separate trees resulting in typical blight infections. This re-isolation of the blight pathogen indicates that the fungus survives and persists on both Chinese and OxO-expressing transgenic chestnut trees, confirming a tolerance mechanism (reduced *impact* of infection) rather than a resistance mechanism involving avoidance or antibiosis (Tiffin, 2000). This relationship is also visible on demonstration trees of an older transgenic event called Darling 4, which have survived for several years despite serious blight infections (Section 10.1 and Figure 10.5.1b). In conjunction with the noted saprophytic survival of *C. parasitica* on Chinese chestnuts and non-chestnut species (Sections 3.1 and 5.2), it is clear that restoring a blight-tolerant American chestnut with OxO should not increase plant pest risks related to presence or survival of *C. parasitica*, because this pathogen is already widespread and endemic on other tree species.

Following the discussion of tolerance terminology in Section 5.1, we have often referred to the function of the OxO enzyme as enhancing “blight resistance” in Darling 58 and other transgenic chestnuts. While this is true in a general sense, the phrase is ambiguous and may be misunderstood. The term “blight” (Section 3.1) can correctly refer to the interaction between *C. parasitica* and a susceptible chestnut tree, but it is also sometimes applied to the physical damage caused to a plant by a pathogen, or to the pathogen itself. In many contexts, the distinction is not important, but in this petition we try to restrict use of the term “blight” to the disease, or the interaction between pathogen and host. Similarly, the general term “resistance” is broad and can be used to describe an array of methods by which a plant can “resist” pest damage (Woodcock *et al.*, 2018; Section 5.1). In the case of the Darling 58 American chestnut, the “resistance” or plant defense mechanism is more specifically based on tolerance (Woodcock *et al.*, 2018), where the pathogen is not harmed or repelled, but instead is tolerated by the host, similar to the interaction in its native environment on Chinese chestnut trees. Therefore, a more precise description of Darling 58 is a “blight-tolerant” American chestnut tree.

6.3.1 Mechanisms of tolerance to chestnut blight provided by OxO

We have observed natural colonization of *Cryphonectria parasitica* on OxO-expressing transgenic American chestnuts (Figure 10.5.1b), but when OxO is expressed at sufficient levels, infections typically form smaller or superficial cankers. We have also repeatedly observed small, non-lethal

cankers on Darling 58 and other OxO-expressing transgenic American chestnuts after intentional inoculations (Figures 8.1.3a and 8.1.3b). Natural blight infections have only rarely been observed on Darling 58 trees due to their age and size to date (the largest Darling 58 trees alive at the time of writing are about 3 years old), but there is no reason to expect that the presence of OxO would prevent initial colonization of the tree by the fungus. Hydrogen peroxide could hypothetically exert preventative antimicrobial properties, but since the H₂O₂ byproduct of OxO activity relies on OA produced by the blight fungus, that is not a logical interaction in OxO-expressing chestnuts (Section 6.3.2). The life cycle of *Cryphonectria parasitica* also does not appear to be disrupted by presence or expression of OxO in the American chestnut. We have observed asexual spore reproduction on OxO-expressing trees, and unlike most susceptible chestnuts, cankers on OxO-expressing trees can persist for multiple years after infection without killing the tree (Figure 10.5.1b). Cankers are only reduced in size and severity (Section 8.1), which allows the tree to continue functioning normally.

Tolerance of transgenic chestnut tissue to OA has been demonstrated by exposing Darling 58 leaf discs directly to oxalic acid, without the blight fungus present (also described in Section 5.4). Related methods have been successfully used to screen for susceptibility to OA-secreting pathogens in other plants, including white bean (Kolkman and Kelly, 2000), peanut (Livingstone *et al.*, 2005), and poplar (Liang *et al.*, 2001). In our first test, chestnut leaf discs (16 mm diameter, n = 9 – 12 per type) were soaked overnight in 50 mM oxalic acid, and imaged on a flatbed scanner for quantification (Figure 6.3.1a, left). Leaf types included T2 transgenic and non-transgenic offspring of Darling 58 (full siblings from the same mother trees; see Section 6.4). A second experiment also included Qing Chinese chestnut controls (Figure 6.3.1a, right). Necrosis due to oxalic acid damage is clearly visible as a brown area around the cut edge of each leaf disc (Figure 6.3.1a, photo insets), while green areas are healthy tissue (unaffected by oxalic acid). Green (non-necrotic) area of each disc was calculated from scanned images of all leaves, and mean area of each type was compared with Tukey's HSD test ($\alpha=0.05$; not shown on graphs). In both experiments, non-transgenic American chestnut tissues (full siblings from the same cross) are visibly susceptible to damage from oxalic acid (nearly no remaining green tissue), while transgenic offspring from Darling 58 show significantly less damage ($p < 0.001$). Necrosis on Chinese chestnut leaves was not significantly different than transgenic T2+ leaves ($p > 0.9$). Results are similar to those seen after similar tests on other OxO-expressing plants: reduced necrosis from exogenous OA has been reported on OxO-transformed soy (Yang *et al.*, 2019), rice (Molla *et al.*, 2013), and tomato (Walz *et al.*, 2008), and results from other OxO-expressing chestnut events are described in Section 10.5. These leaf disc experiments clearly demonstrate that OxO accomplishes the purpose of protecting Darling 58 tissues from damaging effects of OA.

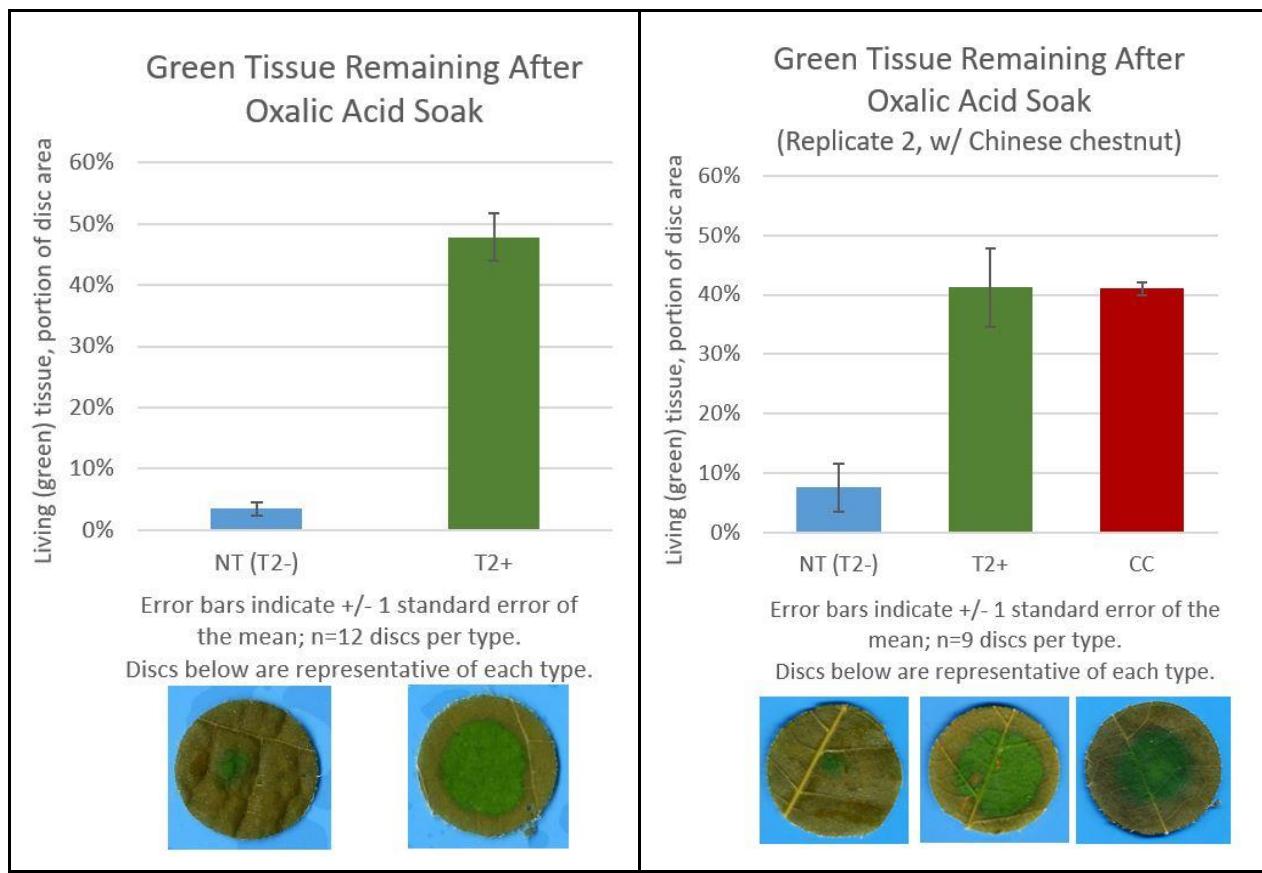


Figure 6.3.1a. Mean necrosis on leaf discs after soaking in oxalic acid for ~20 hours. T2+ (green columns) are T2 transgenic offspring of Darling 58 American chestnut; NT (blue column) are non-transgenic full siblings from the same mother trees; CC (red column) is Chinese chestnut. Example discs with necrosis similar to the mean for each type are shown under each column for reference. Some of these data are also shown in Figure 5.4a.

6.3.2 Potential effects of H₂O₂ from OxO transgene activity in chestnut

As described in Section 4.3, plants naturally produce hydrogen peroxide in response to many diverse stimuli using various mechanisms. Oxalate oxidase as naturally found in monocots and other plants can be one mechanism for H₂O₂ production, which is expressed during germination in wheat but can be induced by pathogen infection at later stages (Lane, 2002; Section 4.1). Plants also have a variety of mechanisms for degrading H₂O₂ before it accumulates to harmful levels, so non-transgenic trees already produce (and degrade) H₂O₂ independent of OxO. Therefore, the hydrogen peroxide byproduct of OxO activity in Darling 58 is unlikely to pose an increased plant pest risk compared to traditionally bred chestnuts.

It is possible for OxO to produce hydrogen peroxide via a separate enzymatic pathway called superoxide dismutase (Woo *et al.*, 2000), but this only occurs at neutral to high pH (~7.5) such as found in corn leaves (Ramputh *et al.*, 2002), not the acidic conditions found in chestnut blight canker margins (pH ~2.8) or stems (pH ~5.5) (McCarroll and Thor, 1978).

Hydrogen peroxide (produced by OxO or other mechanisms) can also function as a signal to induce other plant defense responses (Section 4.3.2; Molla *et al.*, 2013), and it is possible that this is one

of the mechanisms occurring in Darling 58. However, we have no evidence of such effects, but if present, the effect would be secondary and not likely a significant factor in achieving blight tolerance. We also have no evidence that H₂O₂ produced by the degradation of OA directly kills the fungus. However, the observed oxalic acid tolerance in Darling 58 (Figure 6.3.1a), and continued survival of the fungus after inoculations (Section 8.1), both support the hypothesis that oxalic acid degradation is the primary OxO mechanism responsible for the blight tolerance in Darling 58.

One important pathway by which plants produce and respond to H₂O₂ is the hypersensitive response (HR) (Levine *et al.*, 1994; Section 4.3). HR genes that produce H₂O₂ or other reactive oxygen species (ROS) have been reported specifically in chestnut, where they are upregulated in response to blight (Barakat *et al.*, 2012) and other fungal interactions (Baptista *et al.*, 2007). A European-Japanese hybrid chestnut produces H₂O₂ in response to insect invasion (Dini *et al.*, 2012; Section 5.5), possibly as a product of enzymatic activity of a germin-like protein such as OxO. Therefore, H₂O₂ is generated in non-transgenic chestnut trees through various endogenous mechanisms under similar stimuli (i.e. blight infection) by which it would be produced via OxO in Darling 58 chestnuts (Section 5.4).

Some pathogenic fungi produce H₂O₂ naturally or manipulate plants' H₂O₂ production mechanisms to their own benefit (Asselbergh *et al.*, 2007). Necrotrophic fungi (Section 5.2), which subsist on dead plant tissue, can particularly benefit from H₂O₂-killed plant cells (Kumar *et al.*, 2001; Shetty *et al.*, 2007). Oxalic acid (OA), as produced by *Cryphonectria*, *Sclerotinia*, and other fungi, reduces photosynthetic efficiency (Fagundes-Nacarath *et al.*, 2018), and naturally manipulates the plant's ROS to initiate programmed cell death (Kim *et al.*, 2008; Williams *et al.*, 2011) to directly benefit the fungi. Not surprisingly, some of these same pathogenic fungi also have endogenous mechanisms for degrading H₂O₂, including *Botrytis* (Gil-ad *et al.*, 2000; Schouten *et al.*, 2002, Lyon *et al.*, 2007), *Sclerotinia* (Williams *et al.*, 2011), and *C. parasitica* (Nuskern *et al.*, 2017). Mycorrhizal fungi can also tolerate, manipulate, and produce H₂O₂ as part of the recognition process as they form relationships with plant roots (Salzer *et al.*, 1999; Baptista *et al.*, 2007; Zhang, R.-Q. *et al.*, 2013). Therefore, direct fungicidal effects of H₂O₂ at biologically realistic concentrations are not likely to substantially interfere with existing interactions between chestnut trees and fungi.

Given the need for the OA substrate, the limited quantity of H₂O₂ produced by OxO, and its rapid degradation in plants (Section 4.3), OxO-generated H₂O₂ in Darling 58 chestnuts would essentially only be found immediately surrounding cankers where the blight fungus produces OA, not in leaves, flowers, nuts, or roots. And given that there is a 1:1 stoichiometric correlation between moles of OA degraded and moles of H₂O₂ produced, molar H₂O₂ concentrations produced by oxalate oxidase could never surpass oxalate concentrations. The relative instability of H₂O₂ compared to oxalate suggests H₂O₂ concentrations at any given time would be substantially less than final OA concentrations observed in chestnut canker tissue.

We have not specifically measured concentrations of H₂O₂ in transgenic (or non-transgenic) chestnuts. However, we know that any pre-existing H₂O₂ must be found at extremely low concentrations in both transgenic and non-transgenic tissues, because chestnut tissue (leaf, stem, root, flower, and cotyledon tissues have been tested) show no observable color change when

placed in a solution that produces a dark blue-black staining in the presence of H₂O₂ (see the description of the negative control in the histochemical OxO activity assays, Section 7.4.1). Staining is only observed when OA is added to the solution with transgenic tissue, in which case H₂O₂ is produced by OxO activity.

It is possible to roughly estimate *maximum* quantities of H₂O₂ that could be generated by OxO based on the quantity of oxalic acid substrate found in blight-infected chestnut tissues. Oxalic acid production by *C. parasitica* has been measured per gram of fungal tissue *in vitro*, but it is difficult to translate artificial media conditions to concentrations produced in cankers. Bennett and Hindal (1989) report a wide range of 5 – 250 mg OA per gram of dry mycelia, depending on the fungal strain and C/N sources, while Havir and Anagnostakis (1983) report 10 – 65 mg OA per dry weight of cultured fungus after 10 days. We are aware of two publications that report *in vivo* OA concentrations in blight-infected chestnut stem tissue, which subsequently allows us to calculate theoretical maximum H₂O₂ concentrations that could result from OxO activity. Gottstein *et al.* (1989) reported 5.9 mg OA per gram of infected fresh tissue, and McCarroll and Thor (1978) reported 9.3 mg per gram of dry tissue. Chestnut wood moisture content of ~45% (Forest Products Laboratory, 1931) would account for most of the difference between these reports in fresh and dry tissue; 9.3 mg per gram of *dry* tissue would be equivalent to ~5 mg per gram of *fresh* tissue. These measurements reflect accumulated OA at the edge of established cankers, presumably over the course of several weeks of blight infection. Using the higher quantity for example, 5.9 mg of OA is 65 millimoles (formula weight 90.03 g/mol), so if all the accumulated OA in a gram of infected tissue were instantly degraded by OxO, 65 millimoles of H₂O₂ would be generated (or approximately 2.1 mg, based on a formula weight of 34 g/mol). 2.1 mg H₂O₂ per gram of tissue is about 0.2% by weight (but this is not directly equivalent to percent by *volume* concentrations, such as those reported for liquid solutions of H₂O₂). This represents a theoretical maximum cumulative quantity of H₂O₂ produced by OxO per gram of canker margin tissue in a transgenic chestnut tree.

As described previously, OxO in the tree would be continually degrading OA as it is produced by the blight fungus, and the H₂O₂ itself would continually degrade (due to inherent chemical processes or active plant or fungal scavenging mechanisms; Section 4.3), so H₂O₂ would logically never accumulate to the final concentrations reported above. Additionally, since OxO expression in Darling 58 has been observed to limit the size of cankers (Section 8.1), and *C. parasitica* produces the highest quantities of OA during stages of rapid canker growth soon after initiating an infection on a susceptible chestnut (Havir and Anagnostakis, 1983), total OA production at any given infection site on a transgenic tree may be less than that on a blight-infected non-transgenic tree. Thus the maximum H₂O₂ concentrations estimated above are likely much higher than would be found in real-world conditions in a transgenic tree.

A careful consideration of the nature of H₂O₂, its presence in non-transgenic chestnuts, and its limited production by OxO in transgenic chestnuts suggests that H₂O₂ generated by OxO is unlikely to lead to fundamentally different conditions or processes than those already found in non-transgenic chestnuts. H₂O₂ is already induced in chestnuts by fungal pathogens and other stimuli, and plants already have mechanisms for quickly degrading excess H₂O₂ *in situ*. Most importantly, H₂O₂ generated by OxO is unlikely to lead to off-target metabolic effects that could pose an increased plant pest risk compared to non-transgenic chestnuts.

6.3.3 Stability of the OxO tolerance mechanism in Darling 58

This subsection addresses a potential concern that the chestnut blight fungus could evolve to overcome tolerance conferred by an oxalic acid tolerance trait (Section 5.3), and explains why chestnut blight tolerance conferred by OxO expression is unlikely to break down or fail if deployed to the natural ecosystem. The continuing presence of *C. parasitica* on Darling 58 trees (Sections 6.3 and 8.1) has the unique benefit of reducing selective pressure for the fungus to overcome the action of the OxO enzyme (Section 5.3). Darling 58's tolerance mechanism, in which the tree continues to serve as a host for fungal colonization and reproduction, and the continued presence of non-transgenic trees in the landscape (including Darling 58 offspring that do not inherit the transgene), specifically demonstrate a lack of novel plant pest risks, even toward the chestnut blight pathogen.

As *C. parasitica* encounters the oxalate oxidase enzyme in a transgenic tree, the fungus is not killed, rather it continues to exist in saprophytic form, from which it can be easily re-isolated in artificial culture (Figure 10.5.1c; confirmed by morphological evaluation and by re-inoculation of other chestnuts resulting in typical blight infections). Figures 8.1.3a and 8.1.3b also show substantially reduced but still-visible cankers on Darling 58 American chestnuts. In a forest setting, if such encounters occur after proliferation of the OxO tolerance trait, it is expected that the effect will be similar, i.e. the blight pathogen will continue to exist in saprophytic form. Importantly, there is no selection pressure on the fungus to exert necrotrophic or parasitic effects on any living substrate in order to survive (Section 5.3).

It is worth noting that due to the inheritance of OxO by at most ~50% of offspring from hemizygous transgenic parent trees crossed with non-transgenic trees (Section 6.4), there will essentially always be non-transgenic American chestnuts present, even in hypothetical restoration scenarios where transgenic chestnut trees are widely planted. While blight-tolerant Darling 58 trees allow the fungus to survive, these non-transgenic offspring will likely persist indefinitely in reduced form much as American chestnuts do today, serving as additional fully susceptible refuges (Meihls *et al.*, 2008) for blight populations, further reducing the likelihood that the chestnut blight pathogen will evolve resistance to the OxO trait.

6.4 OxO inheritance by Darling 58 offspring (T1 and T2 generations)

The gene for OxO can be passed down to the offspring of a transgenic tree when crossed with another chestnut tree. American chestnut is monoecious and predominantly self-infertile; it takes two trees to produce viable nuts (Section 2.3.1). Since only one copy of the transgene is present on one chromatid in Darling 58 (known as a hemizygous state; Section 7.2.2), roughly half the offspring will receive the chromatid containing the transgene as the chromosomes separate during meiosis. The blight tolerance trait is thus inherited by about half the offspring from a cross with one hemizygous transgenic parent. This is dramatically visible on blight-infected full-sibling pairs when one individual has inherited the transgene and one has not (Figures 8.1.3a and 10.5.1d). If two hemizygous transgenic Darling 58 offspring were to eventually cross with each other, about 25% of their offspring would be homozygous, and 25% would be non-transgenic. This presence of non-transgenic offspring will be a useful conservation tool that allows production of the original wild-type trees long into the foreseeable future.

Controlled pollinations with transgenic chestnut pollen have been performed according to permit conditions in permitted field plots for several years, though some of these have been with legacy events not being submitted for regulatory consideration (Section 10.3). We have generally observed that approximately half the offspring from these crosses inherit the OxO transgene, confirming expected inheritance patterns as described above. Female flowers must be bagged before they are receptive (“pre-bagged”) in order to prevent pollination by airborne pollen from surrounding chestnut trees; late pre-bagging may result in lower than expected transgene inheritance rates because some of the nuts may have actually been open-pollinated. Regardless of pre-bagging efficiency, all transgenic pollinations and potentially transgenic nuts remain bagged, according to permit conditions, until after they are harvested and moved indoors.

Chi square analyses on inheritance rates of Darling 58 offspring to date have been conducted to test whether inheritance rates were significantly different than the expected 50%. All chi square tests were completed using the CHISQ.TEST function in Microsoft Excel 2013 ($\alpha=0.05$; Table 6.4a). If the resulting χ^2 value is less than 0.05, actual inheritance rates are significantly different than the expected 50%. Inheritance rates significantly less than 50% may indicate pollination by wild-type pollen (see above), while inheritance rates significantly greater than 50% could indicate multiple transgene copies.

Darling 58 pollen was first successfully collected and used in the 2016 pollination season, during which three separate non-transgenic mother trees were pollinated. In 2016, a total of 19 nuts from pollinations with Darling 58 pollen were collected, of which 6 inherited OxO (~32%, $\chi^2 = 0.108$). While chi square analysis did not indicate a statistically significant difference from the expected 50%, this apparently low inheritance rate may be explained by late pre-bagging of female flowers (which could have allowed pollen from other non-transgenic trees to have pollinated the flowers before bagging), but could also simply reflect normal variation in a relatively small sample size. All of these seeds were germinated in a greenhouse after cold stratification, and in the spring of 2017 they were planted outdoors in permitted field locations. Trees from the same crosses (full siblings) that did not inherit the OxO gene were planted nearby. These pairs of T1 transgenic and non-transgenic full siblings have been used for studies described elsewhere in this petition (e.g. Sections 8.1.3, 8.2, and 8.3). Additionally, some of the T1 individuals have been vegetatively propagated via cuttings followed by tissue culture shoot multiplication, so we have included some of these lines (primarily those known as D58+16001 and D58+16020) in various experiments and analyses (e.g. “potted small stems” in Section 8.1.3).

The following year pollen exclusion bags were placed earlier, and more flowers were pollinated with Darling 58 pollen on a total of four mother trees (two of which had also been pollinated in 2016). After harvesting these 2017 pollinations, we tested 40 nuts from Darling 58 pollinations, and determined 24 had inherited the transgene (60%, $\chi^2 = 0.206$). All 40 of these nuts were evaluated for OxO concentration (Section 7.4.2) and/or processed for nutrition testing (Section 8.4.1), so they were not germinated or planted. Remaining nuts from 2017 pollinations were damaged during storage due to equipment failure before they could be tested or germinated.

Pollen from two T1 offspring of Darling 58 trees (known as D58+16001 and D58+16020) was used for pollinations on 15 American chestnut mother trees in 2018, resulting in a second outcross (T2, or OC2) generation consisting of nearly 1600 nuts from American chestnut mother trees (Table

6.4a). These 2018 mother trees included those pollinated in previous years. Transgene inheritance by the T2 generation varied by mother tree from 32 - 49%, likely because some mother trees flowered early and could have been partially open-pollinated before bagging. While transgene inheritance percentages vary somewhat, it is important to note that Darling 58 crosses have never resulted in significantly more than 50% inheritance, which is consistent with a single copy of the transgene as described in Section 7.2.2. Total inheritance numbers from Darling 58 crosses, chi square analyses, and additional details about pre-bagging are shown in Table 6.4a. Additional pollinations with T1 pollen were performed in 2019; inheritance results will be published and/or shared when they are available (testing underway; results anticipated late spring 2020).

Table 6.4a. Chi square analysis of transgene inheritance by Darling 58 American Chestnut offspring. MT = mother tree (non-transgenic pollen recipient tree identified with 2-letter code).

*Field records indicate starred mother trees were pre-bagged after some female flowers may have ripened, possibly resulting in pollination by airborne non-transgenic pollen.

| Generation (year, mother tree) | Number Tested | Observed Transgenic | Observed Non-Transgenic | Expected (50%) | Percent Transgenic | P value from χ^2 table |
|---|---------------|---------------------|-------------------------|----------------|--------------------|-----------------------------|
| T1 (2016, three MTs*; n = 5 – 8 nuts/tree) | 19 | 6 | 13 | 9.5 | 32%* | 0.108 |
| T1 (2017, four MTs; n = 10 nuts/tree) | 40 | 24 | 16 | 20 | 60% | 0.206 |
| T2 (2018, MJ*) | 521 | 184 | 337 | 260.5 | 35%* | 2.04e-11 |
| T2 (2018, FA) | 423 | 203 | 220 | 211.5 | 48% | 0.408 |
| T2 (2018, MA*) | 126 | 47 | 79 | 63 | 37%* | 0.0438 |
| T2 (2018, FC*) | 98 | 42 | 56 | 49 | 43%* | 0.157 |
| T2 (2018, WA) | 88 | 32 | 56 | 44 | 36% | 0.0105 |
| T2 (2018, WZ*) | 57 | 18 | 39 | 28.5 | 32%* | 0.00541 |
| T2 (2018, BA) | 56 | 27 | 29 | 28 | 48% | 0.789 |
| T2 (2018, AC*) | 70 | 30 | 40 | 35 | 43%* | 0.232 |
| T2 (2018, FD) | 39 | 19 | 20 | 19.5 | 49% | 0.873 |
| T2 (2018, 8 other MTs*; n = 3 – 18 nuts/tree) | 70 | 27 | 43 | 35 | 39%* | 0.0558 |
| Total Darling 58 Offspring | 1607 | 659 | 948 | 803.5 | 41%* | 5.63e-13 |

Given the inheritance patterns described above, a unique aspect of chestnut restoration using Darling 58 American chestnut trees is that they will not fully replace blight-susceptible, wild-type American chestnut trees. On the contrary, transgenic chestnuts could actually help preserve non-transgenic trees in American chestnut populations because wild-type offspring will continually be produced, especially given plans to intentionally outcross Darling 58 with diverse surviving wild-type trees (Section 11.2). This could benefit the conservation of *C. dentata* as a species because regardless of how long these blight-tolerant American chestnut trees live, some of their offspring will still be non-transgenic, which conserves genetic diversity and keeps possibilities open for future research and restoration efforts.

7.0 Genetic analysis and molecular characterization of Darling 58

Transgenic American chestnut tissues expressing the gene for OxO may be quickly differentiated from non-transgenic tissues via colorimetric enzyme assay (Section 7.4.1). However, this method is not quantitative, and amplifying DNA via polymerase chain reaction (PCR) offers the opportunity to detect the transgene DNA directly (Section 7.2.1). Darling 58 transgenic tissues have been further characterized using quantitative PCR (qPCR, Section 7.2.2), genome walking (Section 7.2.3), reverse transcription qPCR (RT-qPCR) to quantify mRNA transcript levels (Section 7.3), and by quantitative enzyme assays which detect byproducts of the oxalic acid degradation reaction (Section 7.4.2).

7.1 p35S-OxO vector

The vector used in the transformation of Darling 58 American chestnuts is known as p35S-OxO (Figure 7.1a; Table 10.1a). This vector contains two genes and their associated regulatory sequences: oxalate oxidase (Section 7.1.1) for blight tolerance, and the selectable marker neomycin phosphotransferase (NPTII, Section 7.1.2). The full DNA sequence of the insert is shown in Appendix III. Note that Darling 58 does *not* contain visual markers (e.g. GFP) or antimicrobial peptides, some of which have been tested previously in transgenic chestnuts (see Section 10 for information on legacy events).

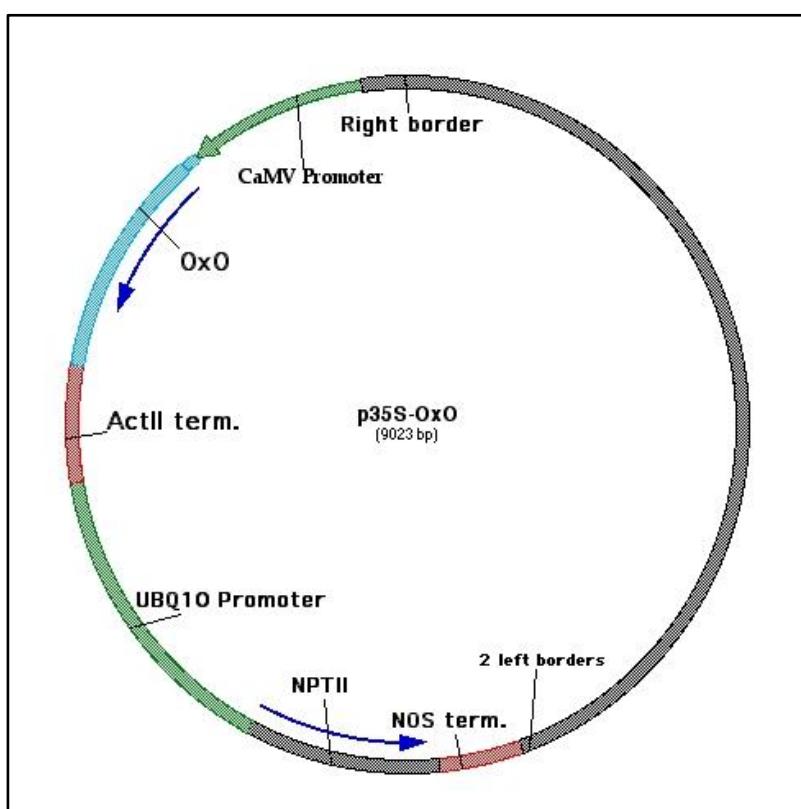


Figure 7.1a. Plasmid construct p35S-OxO as used to transform Darling 58, showing the orientation and relative positions of OxO and NPTII genes and their respective regulatory sequences. Full insert sequence is available in Appendix III.

7.1.1 OxO transgene and regulatory sequences

The gene of interest (gf-2.8, Dratewka-Kos *et al.*, 1989) in this vector comes from *Triticum aestivum* (bread wheat), and codes for the oxalate oxidase enzyme (OxO, EC1.2.3.4) as described in Section 4. Nucleotide and amino acid sequences are shown in Table 7.1.1a. OxO is a germin-like protein that catalyzes the breakdown of oxalic acid (or oxalate) into hydrogen peroxide and carbon dioxide (Figure 4.1a), and protects Darling 58 American chestnuts from harmful effects of chestnut blight, as described in Sections 6 and 8.

*Table 7.1.1a. Oxalate oxidase (gf-2.8) from wheat (*Triticum aestivum*) nucleotide and amino acid sequences (Dratewka-Kos *et al.*, 1989).*

| Sequence Type | Length | Sequence |
|---------------|--------|---|
| Nucleotide | 672 bp | atggggtaactccaaaacccttagtagctggcctgtcgcaatgctttactagctccggccgtctggccaccgaccc agaccctctccaggacttctgtgcggcacccgcacggcaaggcggctcggtaacggcacacgtcaagcccc atgtcgaggccggcgacgacttccttctcgccaagtggcaaggccggcaacacgtccacccgaacggct ccggccgtgacggagactcgacgtggccgagtgccgggtaccaacacgctgggtgttccatgaaccgcgtggactt tgctccggaggcacaacccaccacatccacccgcgtgccaccgagatccgtcatcgatgaaaggtgagctt ctcgatggaaatccttgcagcctcgactccggaaacaagctctactcgagggtggtcgcggccggagagacgttcc tcatcccacggggcctcatgcactccagttcaacgtcgtaagaccgaggccatggcgtctccttcaacagc cagaaccccgattgttgcgtgcccctacgcctcgccatcccaacgcgggtgtccatggcgtctccttcaacagc ggcactccgggtggaggccagggtgttcaagttcaagttgcgtgggttt |
| Amino Acid | 224 AA | MGYSKTLVAGLFAMLLAPAVLATDPPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFL FSSKLAKAGNTSTPNGSAVTELDVAEWPGTNTLGVSMNRVDAPGGTNPPHIHPRATEIGI VMKGELLVGILGSLDSGNKLYSRVVRAGETFLIPRGLMHFQFNVGKTEASMVVSFNSQNPG IVFVPLTLFGSNPPIPTPVLTALKRVEARVVELLSKFAAGF |

The OxO gene in Darling 58 is driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Guilley *et al.*, 1982). Expression under this promoter is considered to be high and present in nearly all tissues, with the possible exceptions of dry seeds, pre-germinated embryos, and pollen, in which expression can be lower or negligible (Mascarenhas and Hamilton, 1992; Sunilkumar *et al.*, 2002; Hraška *et al.*, 2008). Other transgenic plants containing the 35S promoter have previously been granted nonregulated status by APHIS (e.g. creeping bentgrass, petition #15-300-01p; corn, petition #15-124-01p, 13-290-01p; soybean, petition #12-215-01p; and cotton, petition #12-185-01p: USDA-APHIS, 2019). Use of the 35S promoter is so ubiquitous that it is even employed in protocols for identifying genetically modified foods (Fu *et al.*, 2015). Lower levels of OxO expression via other promoters have been shown to result in low to intermediate blight tolerance in American chestnut (Section 10.5.1), so 35S is the most effective promoter tested to date for blight tolerance via OxO.

The OxO gene is followed by the Actin2 (Act2) terminator from *Arabidopsis thaliana*, which was chosen for efficient termination of transcription and addition of a poly-A tail to the mRNA. The

first use of this terminator in chestnut was in a construct known as VspB4, as described by Polin *et al.* (2006).

7.1.2 NPTII selectable marker and regulatory sequences

In the p35S-OxO construct, the selectable marker gene NPTII is controlled by the constitutive promoter UBQ10, which comes from the Ubiquitin 10 gene from *Arabidopsis thaliana*. It is followed by the nopaline synthase (Nos) 3' terminator from *Agrobacterium tumefaciens*, which was chosen to terminate transcription and add poly-A tails to the mRNA.

Expression of NPTII from *Escherichia coli* allows plant tissue to survive in the presence of aminoglycoside antibiotics such as kanamycin; neomycin; geneticin (G418), or paromomycin (Fraley *et al.*, 1986; Velten and Schell, 1985). When expressed in American chestnut somatic embryo tissue, this served as a selectable marker, facilitating development and selection of transformed lines in the presence of paromomycin (Section 6.2). The NPTII gene does not have any reported plant pest characteristics (EFSA, 2007; USDA-APHIS, 2019 (e.g. Apple petition #10-161-01p)), and has been used in several plants previously deregulated by USDA-APHIS (e.g. apple, petition #16-004-01p; rose, petition #08-315-01p; papaya, petition #04-337-01p; corn, petition #01-137-01p; canola, petition #01-206-02p; and cotton, petition #95-045-01p: USDA-APHIS, 2019). The NPTII selectable marker has also been previously granted a tolerance exemption by the EPA (40 CFR § 174.521, 2007). A more thorough list of events containing NPTII which have been submitted to regulatory agencies is maintained by the International Service for the Acquisition of Agri-biotech Applications (ISAAA, 2019).

7.2 Characterization of transgene DNA

As described in the following subsections, the transgene insertion in Darling 58 did not result in any extraneous insertions of vector or other DNA, or deletions of existing DNA compared to the Ellis isogenic line. This is consistent with previous observations on other transgenic plants, which consistently show minimal unintended changes due to *Agrobacterium*-mediated transgene insertions. In contrast, traditional hybrid breeding, other unregulated processes like mutagenesis, or alternative methods like biolistic transformation can result in numerous unpredictable genomic changes (Schnell *et al.*, 2015; Anderson *et al.*, 2016; Liu *et al.*, 2019). Recent research has also shown that widespread insertion of *Agrobacterium* DNA sequences has occurred naturally in many plant species (Matveeva and Otten, 2019). These researchers found that approximately 7% of tested dicot species (including a few species of walnut trees) are naturally transgenic, with some of these species containing intact and expressed genes from *Agrobacterium*.

In addition to the molecular characterization presented below, full genome sequencing of Darling 58 and selected offspring is underway, which should provide further details about the insertion site and also confirm copy number, stable inheritance, and placement of the insert on the physical map of the American chestnut genome. A preliminary screen for 24-mer sequence matches from the *Agrobacterium tumefaciens* T-DNA binary vector backbone showed no matches in Darling 58 (or T1 or T2 offspring), confirming that there were no insertions of DNA from this plant pest

anywhere in the genome⁴. More detailed genome analyses from Darling 58 and offspring will be shared as they become available (anticipated by late 2020). In the meantime, molecular characterization performed to date on Darling 58 is described in the following subsections.

7.2.1 Polymerase chain reaction and OxO gene sequence data

Initial polymerase chain reaction (PCR) analysis of Darling 58 transgenic tissues was performed on DNA from somatic embryo cultures to confirm transgene presence in *Agrobacterium*-free chestnut tissues (Figure 7.2.1a). PCR was performed using LN primers (Table 7.2.1a).

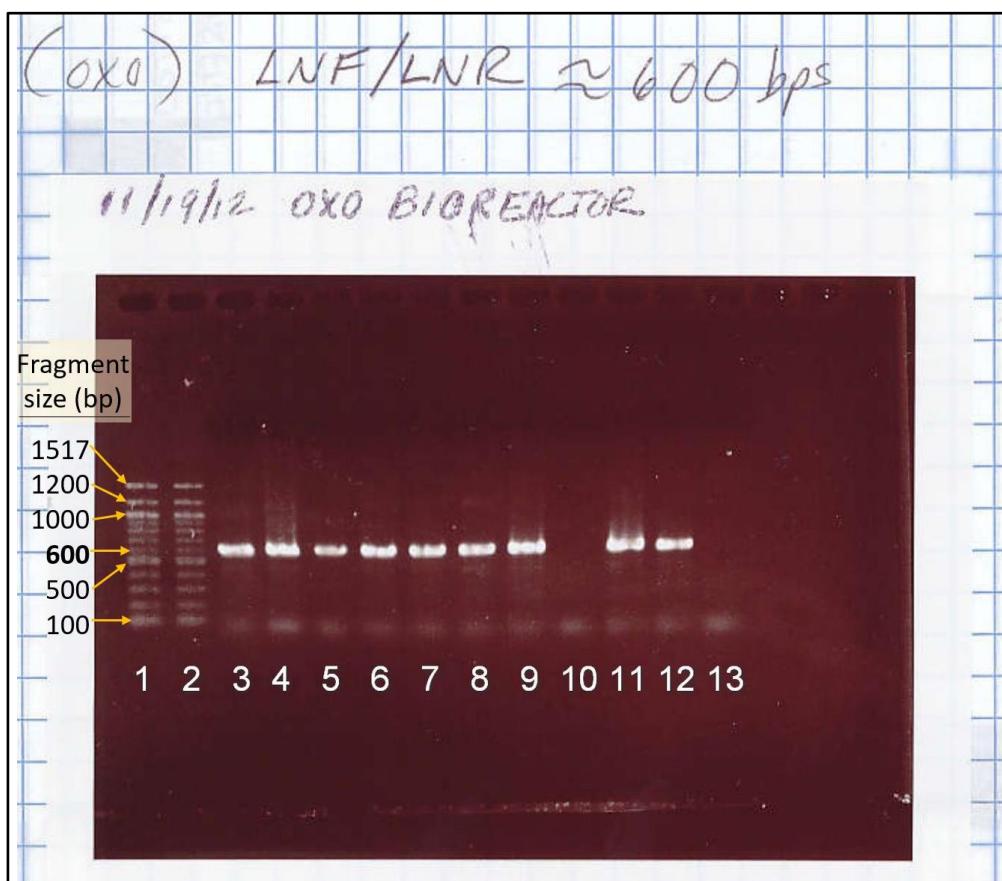


Figure 7.2.1a. PCR amplification of OxO gene fragment (586 bp), on DNA from putatively transformed embryos, including Darling 58 (Lane 11). Lanes 1 – 2 contain 100bp size marker (bands every 100bp and as noted), lanes 3 – 11 are putatively transformed embryo cultures, lane 12 is Darling 4 positive control (Section 10.1), lane 13 is a no-template (negative) control.

PCR was later used to amplify a portion of the coding region of the OxO gene in order to obtain DNA sequence data for this gene after insertion into Darling 58. This was done using genomic DNA extracted from Darling 58 leaves from established shoot cultures, not from embryo cultures as described above. Figure 7.2.1b shows a nucleotide alignment of the OxO coding region, confirming 100% sequence identity among three sources: a portion of the original p35S-OxO

⁴ Personal communication to W. Powell and A. Newhouse from J. Schmutz, HudsonAlpha Institute for Biotechnology, Huntsville, AL, October 3, 2019.

vector sequence, our Darling 58 OxO PCR sequence data, and the original published wheat OxO sequence (Dratewka-Kos *et al.*, 1989). This confirms the OxO DNA sequence as present in Darling 58 is exactly the same as the originally published wheat OxO source.

Figure 7.2.1b. Nucleotide sequence alignment of OxO coding region; see Appendix III for full T-DNA sequence. “p35S-OxO” is the sequence originally used in the Agrobacterium transformation vector, “Sequenced” is sequence data from Darling 58 using “OxO Seq” primers (Table 7.2.1a), “OxO/Lane” is the published nucleotide sequence (Dratewka-Kos et al., 1989; Lane et al., 1993) used to develop the Agrobacterium vector, and “Consensus” is the matching sequence showing 100% identity between all three sources.

Many subsequent PCR experiments have been done using template DNA from Darling 58; additional PCR data are not shown here since other forms of data (copy number, expression, and flanking sequences, see below) serve as further confirmation of transgene presence and integration.

Table 7.2.1a. Primer names and sequences for PCR, qPCR, hybridization probe development (Section 10.4.1), and sequencing.

| Name | Usage | Forward Sequence | Reverse Sequence | Product length |
|-----------|---|--------------------------|--------------------------|----------------|
| AN/AZ | Southern hyb. probe | caacaaccagtgccatagac | tccgttcagtgaaaagaacaa | 966 bp |
| IDT1 | qPCR OxO expression, copy # | cagcgccaaacttggacttgagaa | tgcacttcaggtaacgtcggt | 192 bp |
| GFP I1 | qPCR GFP copy # | atcaaagccaacttcaagacccgc | agggcagattgtgtggacaggtaa | 140 bp |
| Actin | qPCR reference gene | ccttgctggtcgtgatctc | gtctcaagttcctgctcatagtc | 149 bp |
| ef1 | qPCR reference gene | cggttactgagtactagccttg | ctgccgaagaccttattgaaag | 84 bp |
| GAP | qPCR reference gene | gctgcactaccaattgtcttg | tcattgaaggaccatcgacag | 129 bp |
| LN | OxO detection PCR | cagaccctctccaggact | ggcaaacttggacttgag | 586 bp |
| NPTII | qPCR NPTII copy # | ttgtcaagaccgaccgtcc | cttcccgttcaggacaac | 124 bp |
| OxO Seq | Sequencing of OxO coding region | attaaaaaccgcggcaaac | ttcgcgaagacccttcctcta | 622 bp |
| SX58 Up | Sequencing upstream flanking to T-DNA | agcttaggttgggtcaggtca | cgttggctaccgtgtatatt | 557 bp |
| SX58 Down | Sequencing downstream flanking to T-DNA | cgcaatgtatggcattttag | ttgcttcttaaggccattg | 429 bp |

7.2.2 Copy number: quantitative PCR (qPCR)

Transgene copy number is an important consideration for transgene expression, inheritance patterns, and potential use of transgenic plants in future restoration programs. It is not surprising that the majority of American chestnut transgenic events we have transformed with *Agrobacterium tumefaciens* received a single copy of the GOI construct (including Darling 58; see below). *Agrobacterium*-mediated transformation commonly results in single- or few-copy transgenic events, as opposed to biotic systems, in which higher-copy events are common (e.g. Dai *et al.*, 2001; Shou *et al.*, 2004). In the context of possible future chestnut restoration, this means genetically diverse transgenic chestnuts could more easily be included in a restoration project (Section 11.2), since transgene inheritance is simpler and more predictable with single-copy events than it would be with multiple-copy events (Section 6.4).

Transgene copy number in older transgenic events was analyzed with Southern hybridization (Section 10.4.1), but more recently, quantitative (real-time) PCR (qPCR) has allowed copy number

determinations to be carried out more quickly and efficiently (Weng *et al.*, 2004; Yang *et al.*, 2012). Older transgenic chestnut events known from Southern analyses to have one and two copies of OxO (Darling 5 and Darling 4, respectively, Section 10.4.1) were used as controls in qPCR experiments to initially determine copy number on Darling 58.

Genomic DNA for these qPCR experiments was extracted from greenhouse- or field-grown leaves using the Qiagen Plant Mini kit (Qiagen Inc., Germantown MD, USA). At least two separate DNA extractions were performed on different individual plants from each transgenic event and amplified independently to achieve a minimum of two biological replicates for each sample type. SYBR Green reagents (BioRad, Hercules, CA) were used to assemble triplicate qPCR reactions (technical replications) according to the manufacturer's instructions, modified to a final single reaction volume of 9 µL. Amplification was done in a BioRad iCycler-48, and analyzed with BioRad CFX Manager software (v1.6). Initial template concentration, and thus copy number, was calculated with the delta-delta cycle threshold ($\Delta\Delta ct$) method (Pfaffl, 2004). All technical and biological replicates were combined in the $\Delta\Delta ct$ calculation. This calculation essentially compares relative amplification of a target sequence (e.g. part of the OxO gene) to that of one or more reference genes known to have consistent copy number (e.g. Actin, see below).

Primers (Table 7.2.1a) were designed to amplify < 200 bp sections of the OxO and NPTII genes, as well as reference genes Actin and Elongation Factor 1 α (ef1). These reference gene primers, along with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were previously determined to be consistent across various chestnut tissues and efficient (> 95%) in qPCR reactions (Appendix IV). Primers for NPTII were used instead of OxO directly in some copy number tests, since the NPTII selectable marker is on the same construct as OxO and should therefore be present at the same copy numbers. Presence of OxO was also confirmed in separate analyses (e.g. Sections 7.2.1 and 7.4), so NPTII was a legitimate substitution for copy number evaluations.

As shown in Figure 7.2.2a, event Darling 4 has approximately twice the qPCR copy number signal as Darling 5, which correlates with the Southern hybridization data indicating two transgene copies in Darling 4 and one in Darling 5 (Section 10.4.1). Darling 58 and its offspring vary slightly from ~0.4-fold to ~0.6-fold compared to Darling 4, but consistently show about half the copy number of this known 2-copy event. Darling 58 shows a similar copy number to Darling 5, which is known to have a single transgene insert. (Since PCR amplification to a given number of cycles is not a perfectly precise process, especially when comparing relative amplification among multiple primers, there is some variability as seen in Figure 7.2.2a, but all the single-copy events generally show similar ratios compared to the two-copy controls.) Collectively this indicates that Darling 58 has a single insertion of the vector T-DNA. Observed inheritance patterns of no more than 50% transgenic offspring from effectively pre-bagged controlled pollinations (Section 6.4), as expected from a single-copy transgenic parent, also corroborate this conclusion.

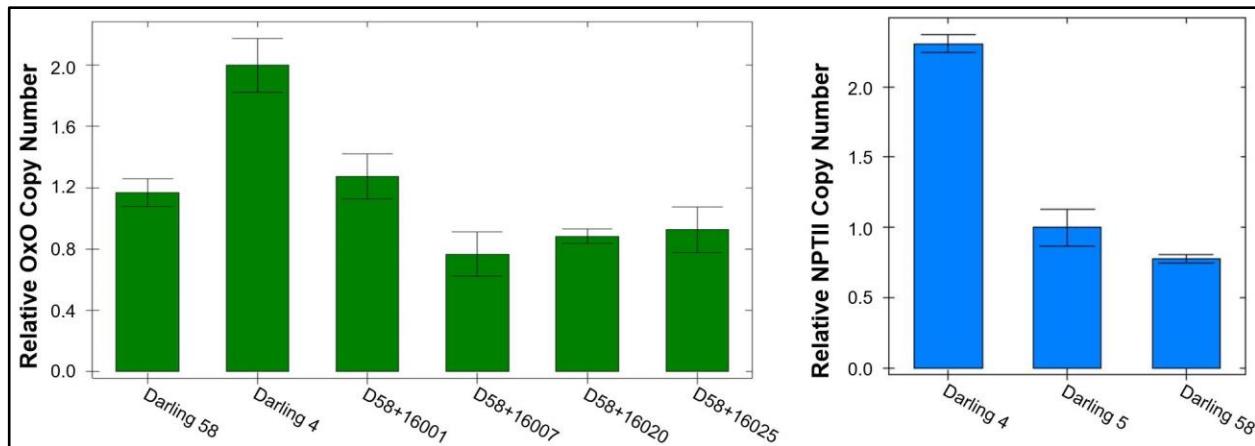


Figure 7.2.2a. qPCR copy number data for Oxo (left) and NPTII (right) genes in Darling 58, compared to known 1-copy (Darling 5) and 2-copy (Darling 4) control events. Reference genes were Actin and ef1. T1 offspring are noted “D58+...”; Darling 58 refers to initial (T0) line.

7.2.3 DNA sequence at insertion site and flanking regions

In order to identify and characterize the insertion site of the transgene vector in Darling 58, we first obtained sequence data from both upstream and downstream flanking regions adjacent to the insertion site, using the GenomeWalker™ Universal Kit (Takara Bio US, formerly Clontech, Mountain View, CA). Sequences from these regions were queried against the Hardwood Genomics Web database (Staton *et al.*, 2015), which provided a relative location in the Chinese chestnut (*Castanea mollissima*) genome. These flanking sequences also align to a portion of Chromosome 7 (formerly Linkage Group G; Kubisiak *et al.*, 2013) of a draft American chestnut (*C. dentata*) genome. A preliminary insert map showing part of Chromosome 7 is shown in Appendix III; further details will be provided when they become available (see introduction to Section 7.2). The American chestnut genome is still in draft form and has not yet been annotated, so comparisons to native genes are based on the Chinese chestnut genome (Staton *et al.*, 2015). Sequence comparisons to Chinese chestnut genome data were performed in collaboration with Dr. John Carlson and Nathaniel Cannon (Penn State University) and Dr. Meg Staton (University of Tennessee). Raw sequencing data for Darling 58 including insert border sequences and flanking genome sequences are shown in Appendix III.

According to flanking sequences derived from genome walking, the p35S-Oxo T-DNA insertion site in Darling 58 is located between positions 10,930 and 11,298 on a 20 kilobase fragment of the Chinese chestnut genome called Scaffold 10296 (Hardwood Genomics Project, 2019; Staton *et al.*, 2015). Figure 7.2.3a shows the relative insert location on this scaffold, the nearest known gene, and the relevant sequence alignments shown elsewhere in this petition.

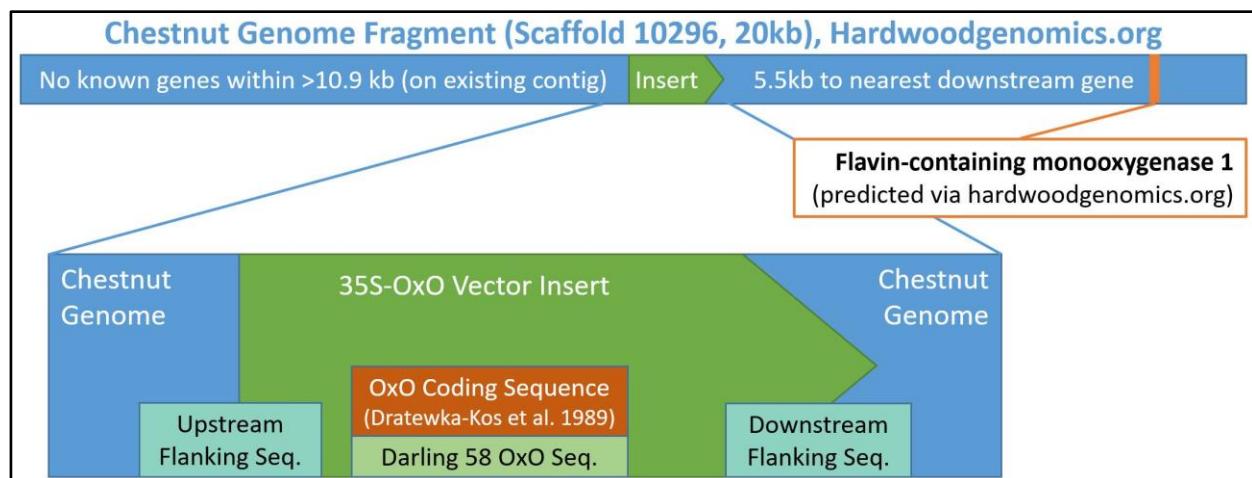


Figure 7.2.3a. Schematic representation (not to scale) of Darling 58 vector insert (green) imposed on Chinese chestnut genome scaffold (blue), including various sources of sequence data (inset boxes) and relative location of the nearest native gene (orange band at top). An alignment of the published OxO Coding Sequence to the actual Darling 58 OxO sequence is shown in Figure 7.2.1b. Insert location in scaffold is based on flanking sequences from Genome Walking (see Figure 7.2.3b).

The insert location in Darling 58 is more than 10.9 kilobases (kb) from the nearest upstream gene, as no known genes are present between the insert and the end of this scaffold based on current annotations. The nearest downstream gene is approximately 5.5 kb from the insertion site based on Augustus predictions (Stanke and Morgenstern, 2005). This downstream gene has been identified as a predicted “Flavin-containing monooxygenase 1” (ID g53985.t1, Cannon and Carlson, personal communication). While the 35S promoter and associated enhancers have been shown to affect expression of nearby host genes in other plants (Wilson *et al.*, 1996; Yoo *et al.*, 2005; Gudynaite-Savitch *et al.*, 2009), this effect is most commonly observed on genes within 3 kb of the 35S sequences and has not been reported to occur beyond 4.3 kb from 35S sequences (Weigel *et al.*, 2000; Tani *et al.*, 2004). Thus expression of an endogenous gene > 5.5 kb away from 35S sequences in the vector insert should not be affected by the insert.

Nucleotide sequence alignments with the vector insert and adjoining genomic flanking sequences are shown in Figure 7.2.3b. The p35S-OxO T-DNA region and chestnut genome sequences (red bars in figure) were aligned to the Darling 58 sequence (blue “Query” bar in figure) with NCBI BLAST tool, and both had > 95% identity (i.e. > 95% of base pairs matching) to the Darling 58 sequence. Note there is only one nucleotide base pair present between the 35S-OxO sequence and the chestnut genome on the upstream side, and no extra base pairs on the downstream side, indicating a lack of extraneous *Agrobacterium tumefaciens* or vector sequences adjacent to the insert in Darling 58 American chestnuts.

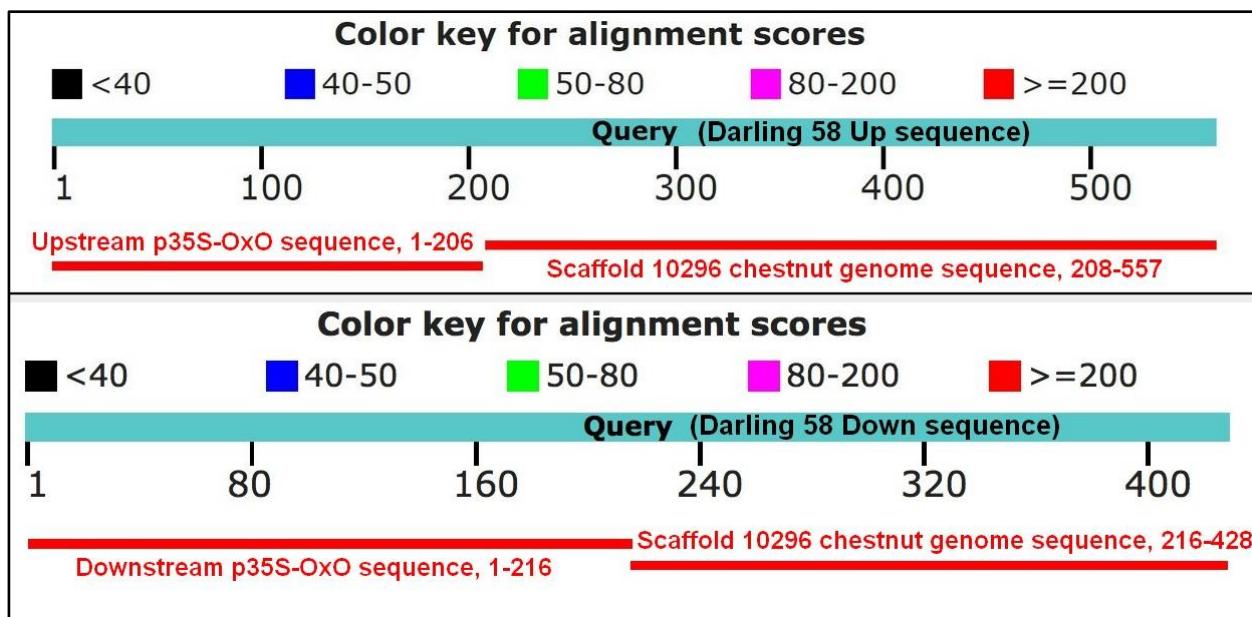


Figure 7.2.3b. Nucleotide sequence alignments of Darling 58 showing upstream and downstream portions of vector insert and flanking regions of the chestnut genome; red bar indicates area with closest nucleotide match (highest possible alignment score). “Query” is sequence data from Darling 58 PCR products using “SX58 Up” and “SX58 Down” primers (Table 7.2.1a) starting inside the p35S-OxO T-DNA region, extending out into the genome. Raw flanking sequence data are shown in Appendix III.

According to PCR and limited sequencing data, when Darling 58 sequences are compared to Ellis 1 genomic DNA, Darling 58 has an inversion of approximately 600 base pairs as shown in Figure 7.3.2c, just outside the left border. This inversion is not near any known genes (see above in this subsection). A more complete understanding of the genome sequence near the insertion site should be elucidated by a whole genome sequence of Darling 58 and offspring, which should be available soon as described above. Changes of this scale, such as inversions, deletions, or rearrangements, are not unusual in either transgenic insertions or natural mutations, and on average rearrangements in transgenic plants may be smaller than changes due to natural rearrangements that occur during breeding (Dane *et al.*, 2015; Anderson *et al.*, 2016; Schouten *et al.*, 2017; Bashir *et al.*, 2018). Therefore an inversion of this magnitude, outside of known coding regions, is unlikely to confer an increased plant pest risk to Darling 58 compared to non-transgenic American chestnut.

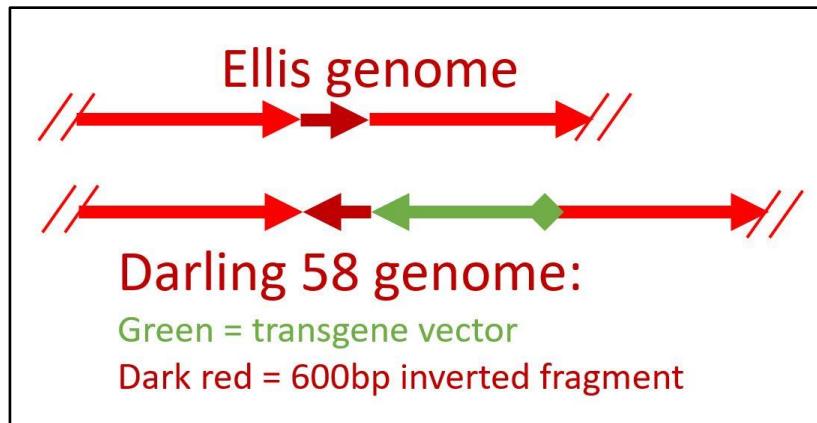


Figure 7.2.3c. Preliminary schematic Darling 58 genome arrangement outside the insert site according to Genome Walking and PCR, showing ~600bp inversion at one end.

7.3 Characterization of transgene mRNA expression by RT-qPCR

Methods for RNA extractions, cDNA synthesis, reverse transcription, and quantitative PCR are based on those described by Zhang, B. et al. (2013). Briefly, tissue was disrupted under liquid nitrogen using a mortar and pestle or freezer mill, total RNA was extracted from young stem tissue using the CTAB method (Chang et al., 1993; Gambino et al., 2008), and cDNA was synthesized from ~0.9 µg total RNA using a Qiagen QuantiTect Reverse Transcription Kit (with the optional DNase treatment step included). Quantitative PCR was performed on cDNA using a BioRad MiniOpticon Real-Time PCR System with Bio-Rad iQ SYBR Super Mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All reactions were performed in triplicate and compared to one or two reference genes. See Section 7.2.1 and Appendix IV for further details on qPCR methods and reference genes.

Zhang, B. et al. (2013) compared Oxo mRNA expression levels with blight tolerance in a variety of Oxo-expressing transgenic lines using leaf assays, and determined that event Darling 215 (AZ-4SX215) showed similar blight susceptibility to the blight-tolerant Chinese chestnut 'Qing'. See Tables 1.3a and 10.1a for details on Darling 215 and similar events, and how they compare to Darling 58. Events with expression levels below those found in Darling 215 showed intermediate levels of tolerance between American and Chinese chestnut controls, and those with higher expression showed increased tolerance. Thus the level of Oxo expression in Darling 215 corresponds to a minimum threshold level of blight tolerance needed for effective functional survival with blight, and is used as a standard for comparison in RT-qPCR. Oxo expression in Darling 4 is also a useful basis for comparison, since we know that Darling 4 can survive natural blight infections for at least several years (Figure 10.5.1b). While Oxo expression in these legacy events is described in more detail in Section 10, they are included here to provide some context for Darling 58 expression measurements, which would otherwise be hard to interpret given a lack of endogenous Oxo expression in American chestnuts.

As shown in Figure 7.3a, stem expression of oxalate oxidase mRNA in Darling 58 (and Darling 58 transgenic offspring) is higher than the minimum threshold established by Darling 215. This is based on woody stem tissue collected from field-grown trees in their second growing season. Expression was tested again on leaf tissue from additional Darling 58 T1 offspring, as well as

Darling 4 (Figure 7.3b). Leaf expression in Darling 4 may not directly correlate to whole-tree blight tolerance, since the *vspB* promoter in that event (Section 10.4) expresses more in stems than leaves, while the 35S promoter in Darling 58 apparently expresses more in leaves than stems (Figure 7.4.2a). Detailed qPCR expression data from both experiments are shown in Appendix V.

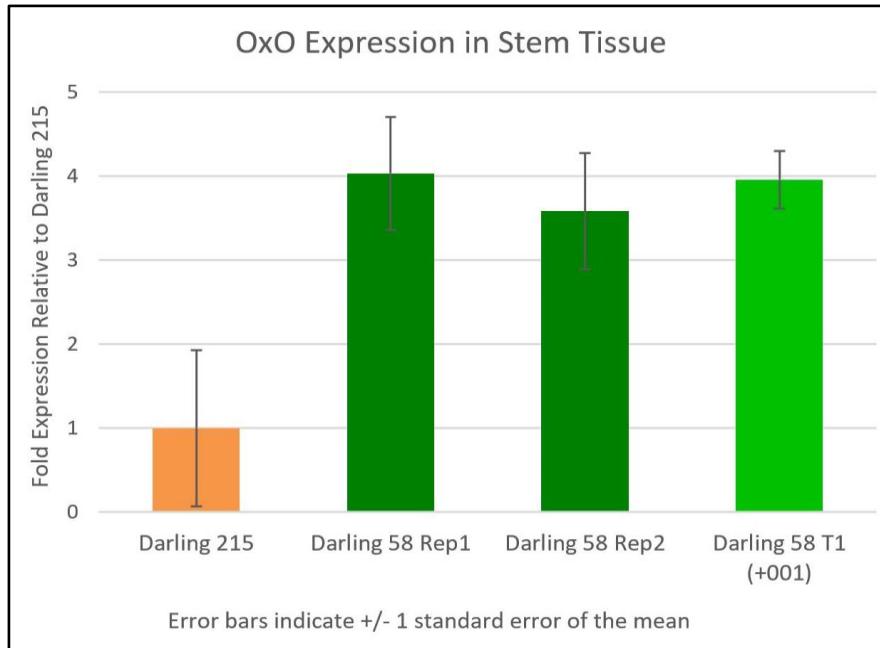


Figure 7.3a. Reverse-transcriptase quantitative PCR (RT-qPCR) determination of OxO expression in woody stem tissue relative to Darling 215. This experiment included Darling 215 (Section 10.1), two replicate individuals of Darling 58, and a Darling 58 T1 transgenic offspring (D58+16001, Section 6.4). Reference gene was Actin. Raw data are shown in Appendix V.

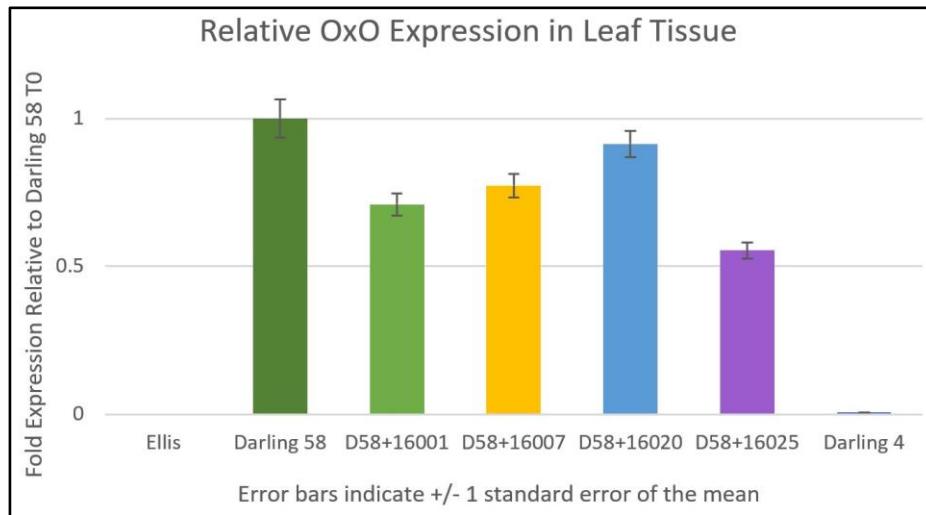


Figure 7.3b. Repeat of reverse-transcriptase quantitative PCR (RT-qPCR) determination of OxO expression in leaf tissue, including Ellis (no expression), Darling 58, four separate T1 offspring (labeled D58+16...), and Darling 4 for comparison. Reference genes were Actin and ef1. Error bars indicate one standard error of the mean of three technical replicates. Raw data are shown in Appendix V.

7.4 OxO enzyme activity and quantification

7.4.1 Histochemical assay

Presence or absence of OxO activity (and therefore the presence of the transgene) in American chestnut tissues can be determined quickly using a histochemical assay adapted from Dumas *et al.* (1995). This assay relies on hydrogen peroxide, produced by OxO in presence of oxalic acid, oxidizing 4-chloro-1-naphthol to produce a dark precipitate that indicates OxO activity (Figure 7.4.1a). The simplicity and reliability of this assay are demonstrated by the use of OxO as a reporter gene in other plant transformation systems (Simmonds *et al.*, 2004; Teng *et al.*, 2018).

To perform the OxO histochemical assay, two tissue pieces are collected from a single source and placed into two separate tubes (Leaf, stem, and nut tissues are discussed in this petition, but this assay should be usable on any living, light-colored tissue type.). The two respective tubes have a histochemical assay solution (Dumas *et al.*, 1995), one containing the oxalic acid substrate ("+"), and one lacking this substrate ("−"). The tissues are incubated for at least 10 minutes (up to 24 hours, depending on tissue type) at room temperature and then visually examined and/or photographed. If the OxO enzyme is present and active in the tested tissues, oxalic acid in the "+" tube is degraded to hydrogen peroxide, which forms a dark blue/black precipitate on the edges of the tissue in that tube (Figure 7.4.1b). Because the reaction relies on generation of hydrogen peroxide (Figure 7.4.1a), and assumes the tissue doesn't contain endogenous oxalic acid, a no-substrate control ("−" tube without oxalic acid) is included for every sample to ensure no extraneous H₂O₂ or OA gives a false positive. This assay has also been used to test OxO in native sources, such as spatial expression of OxO in wheat (Caliskan and Cuming, 1998).

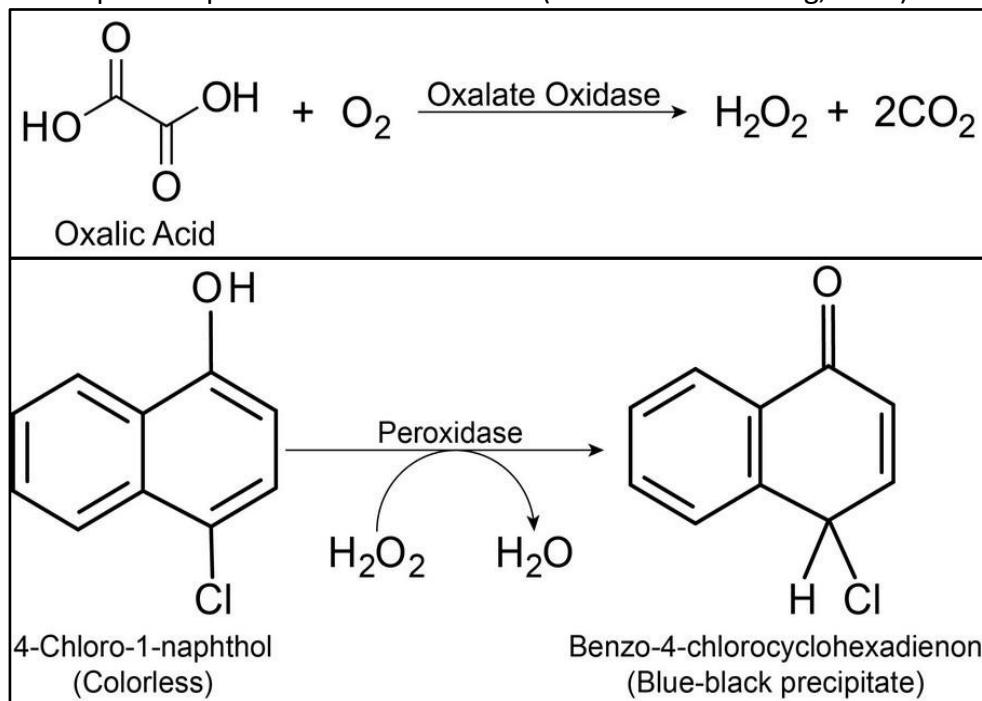


Figure 7.4.1a. Oxalate oxidase mechanism of action (top), and OxO staining reaction based on presence of hydrogen peroxide (bottom).

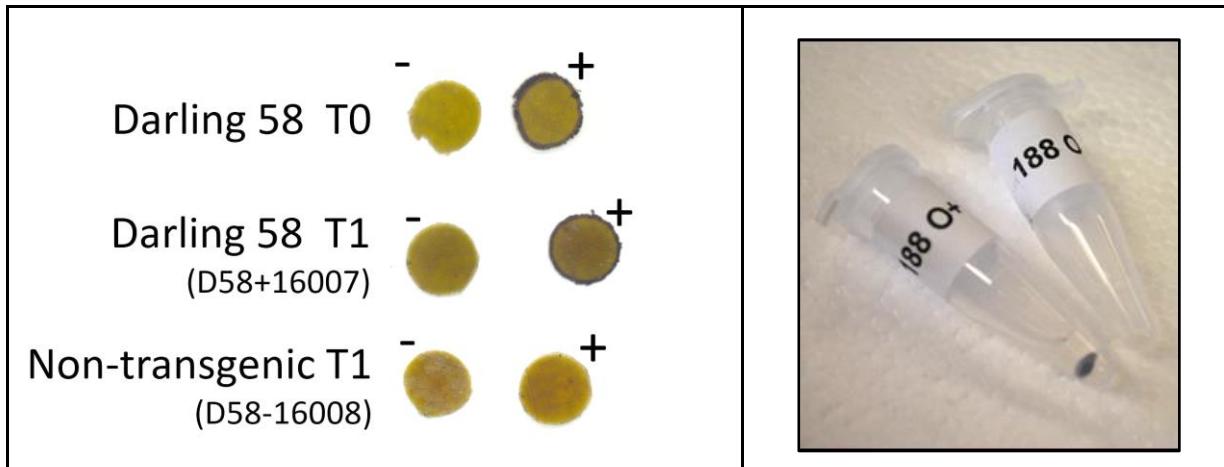


Figure 7.4.1b. Oxalate oxidase (OxO) histochemical assays: dark staining at edges of “+” treatment, and a lack of staining on the “-” treatment, indicates oxalate oxidase activity. At left, 8mm leaf discs: Darling 58 T0 is the original lab-generated clonal line, Darling 58 T1 is a transgenic offspring of the original line, and non-transgenic T1 is a Darling 58 offspring that did not inherit the transgene. At right, Darling 58 T1 transgenic nut tissue (Section 6.4).

7.4.2 Quantitative colorimetric OxO assay

While the histochemical assay above offers a quick qualitative test for OxO activity, a slower quantitative assay is useful for accurately determining OxO concentrations in various tissues. OxO quantities were determined in transgenic American chestnut tissues using natural sources of OxO as comparisons. This was done by measuring hydrogen peroxide output as a function of OxO quantity using purified OxO from barley (*Hordeum vulgare*) to create a standard curve (method adapted from: Sugiura *et al.*, 1979; Zhang *et al.*, 1996; and Li *et al.*, 2015). Barley OxO was used as a control because it was the only purified OxO enzyme commercially available at the time; it has > 96% AA identity compared to wheat OxO (Lane *et al.*, 1991) and has been observed to have the same enzymatic process, so it is the most appropriate control for this use.

Root, stem and leaf tissues of transgenic chestnuts, and food grain sources, were ground separately in the presence of liquid nitrogen in a SPEX 6870 freezer mill (SPEX SamplePrep, Metuchen, NJ), lyophilized, and stored at -80°C. Lyophilized tissue was homogenized in a DNA lysing matrix tube (MP Biomedicals, Santa Ana, CA) in the presence of 40mM succinate activity buffer (Zhang *et al.*, 1996) and diluted if necessary for colorimetric output to fall within the range of a standard curve. Oxalate oxidase quantities in tested tissues (Figures 7.4.2a) were calculated based on the known concentrations of purified OxO in this standard curve.

Quantification of transgenic nut tissue was done using cotyledon tissue excised from recently harvested Darling 58 T1 seeds. Chestnuts from transgenic crosses were surface sterilized by dipping into 50% ethanol and drying in a laminar flow hood. Cores (1.5 mm diameter) were taken from each nut using a double bevel bone marrow needle, taking care to avoid the top of the nut where the embryo is located. The core site was then sealed with 100% silicone adhesive to reduce the chance of infection inside the nut. When the adhesive dried each nut was put back in a labeled plastic bag with damp sphagnum moss and stored at 4°C until germination (typical cold

stratification conditions). Similar coring methods were used to identify transgenic nuts prior to nutrition and composition analyses (Section 8.4).

We removed two 0.5 mm long sections from each core for the histochemical oxalate oxidase assay to determine which chestnuts inherited the transgene (Figure 7.4.1b, right). The remainder of each nut core was stored at -80°C until use in the quantitative assay. Once transgenic nuts were identified, the remainder of the cores from transgenic nuts were pooled and lyophilized.

As shown in Figure 7.4.2a, nut tissue from four different mother trees (Section 6.4) consistently expressed less OxO than leaf or stem tissues, which is not surprising given documented expression patterns of the 35S promoter (lower in dormant seeds than actively growing tissues; Section 7.1.1). It is possible that OxO expression would increase when the nut becomes more physiologically active during germination, but the sampling time for this experiment (soon after ripening/harvest) reflects the time when most people or animals would likely be consuming or interacting with nuts. Root tissue was not significantly different than nut tissue, but roots expressed less OxO than stems, which in turn expressed less than leaves ($p < 0.05$; overall range ~0.3 - 1.1 ug OxO per mg dry weight tissue). Statistically significant differences were identified with a Tukey's HSD test ($\alpha=0.05$). Other processed food grain sources with endogenous OxO activity ranged from 0.003 to 0.008 ug OxO per mg dry weight tissue (Figure 7.4.2a, bottom). All food grain sources showed more than an order of magnitude less OxO than the transgenic chestnut tissues, and it was not important to compare OxO quantities among the various food sources, so statistical comparisons were not completed on the food sources. The food samples represent dried/processed food products most commonly used for baking or brewing; germinating (sprouted) grains sometimes used in specialty food products would likely have higher OxO activity (Lane et al., 1991). It is expected that processed and stored food grains would have lower activity than fresh chestnut tissues, much as transgenic chestnut leaves lose OxO activity when they dry (Section 9.4). Potential consumption of (or exposure to) various quantities of OxO is discussed in relation to dietary exposure and toxicity in Sections 8.4.3 and 8.4.5.

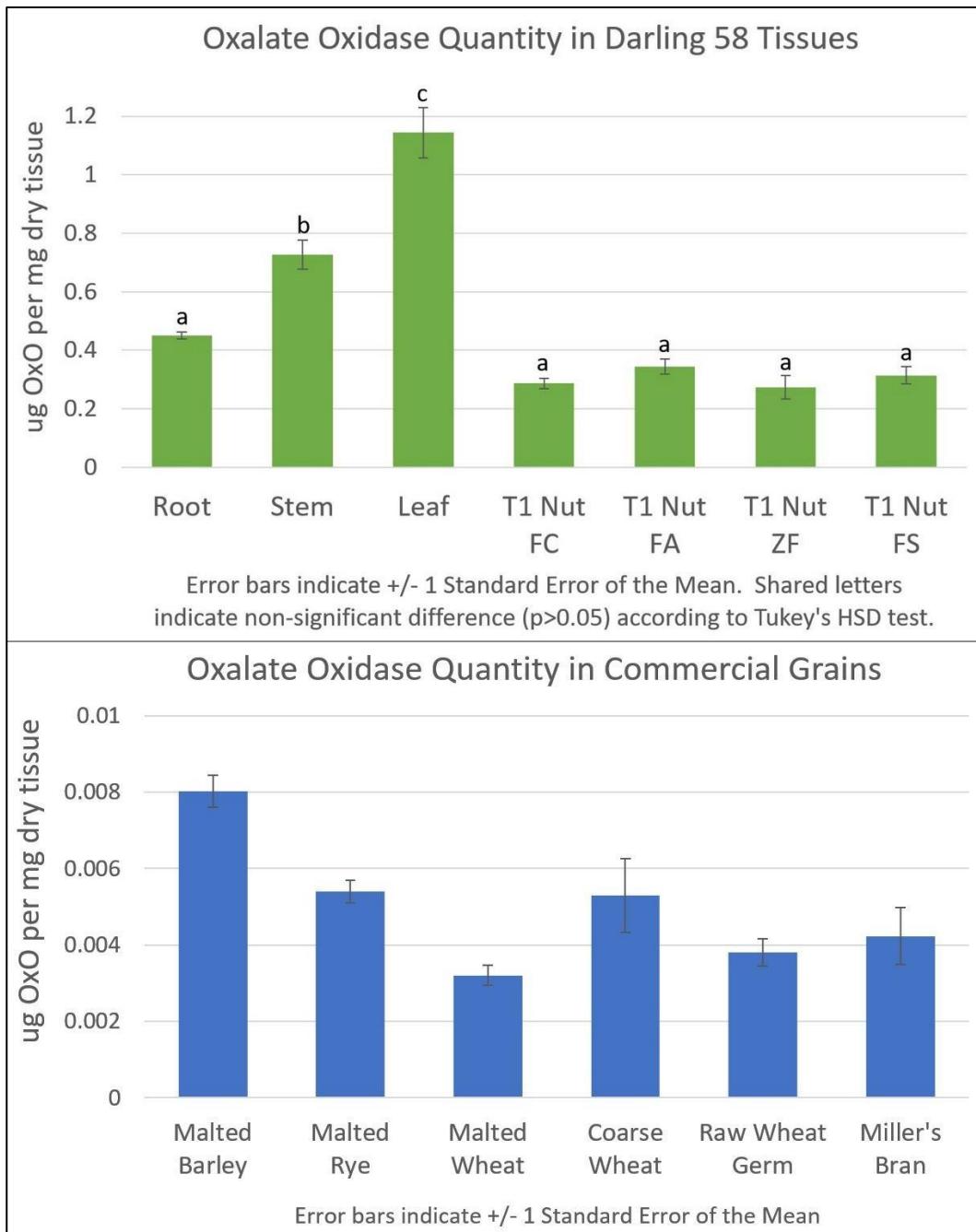


Figure 7.4.2a. Oxalate oxidase quantities (μg of Oxo/mg of dry weight tissue) in Darling 58 tree and nut tissues (top) and commercially available food products (bottom). “T1 Nut” samples are from transgenic nuts from different mother trees (each mother tree represented here by a two-letter code). Values for root, stem, and leaf tissues are means of 6 replicate samples, values for nut tissues are the means of 4 – 6 replicate samples. Note that the y-axis of the bottom graph is on a different scale than that of the top.

8.0 Phenotypic characteristics of Darling 58 American chestnut

This section provides a description of the phenotype of Darling 58 American chestnuts as compared to the Ellis isogenic control and other members of the genus *Castanea*. Phenotypic characteristics described in this section (and environmental interactions, Section 9) have been evaluated as compared to non-transgenic and traditionally bred controls in both field (Appendix I) and laboratory or greenhouse experiments. Additionally, data from similar transgenic events (Section 10) provide further evidence that the presence of OxO or the transformation process does not significantly affect growth or performance of transgenic American chestnuts. The data support the conclusion that the Darling 58 chestnut is no more likely to pose a plant pest risk than comparators such as the Ellis isogenic line, full-sibling controls, or traditionally bred American chestnuts. The most substantial difference is oxalic acid tolerance that allows the tree to coexist with the blight fungus. Phenotypic and environmental interaction characteristics of Darling 58 American chestnut were evaluated to assess potential plant pest risks, and more generally, to assess environmental safety of this potential restoration material. Results from these tests provide no indication that Darling 58 American chestnuts possess increased weediness characteristics, or susceptibility or tolerance to specific abiotic stressors, arthropods, or diseases (other than chestnut blight, discussed previously) compared to controls.

8.1 Blight tolerance

We have used several tests to assess blight tolerance on various chestnut tissues and trees, depending on the age and size of material available. This section describes intentional inoculations on Darling 58 tissues and trees using the chestnut blight pathogen *Cryphonectria parasitica*. Results of inoculations and natural blight infections on older OxO-expressing transgenic chestnut events are described in Section 10.5.1. Further tests on additional outcross generations of Darling 58 offspring will be performed when these trees are large enough to inoculate, and we will continue to share and/or publish results as they become available.

8.1.1 Leaf inoculations

Our initial process for assessing blight tolerance is a leaf inoculation assay (Zhang, B. *et al.*, 2013; Newhouse *et al.*, 2014a). This assay involves making a shallow wound along the midvein of an excised leaf, applying a plug of agar with *C. parasitica* against the wound, and enclosing inoculated leaves in a humid chamber. After storing for 5 – 7 days in the dark at room temperature, necrotic area is measured and compared to known susceptible and resistant controls. This assay can be used on plants less than a year old, without causing permanent damage, before they are planted outside. It is considered a preliminary screen, as it does not always distinguish fine differences in susceptibility (LaBonte *et al.*, 2016). However, it is useful for identifying events with relatively high, intermediate, or low levels of blight tolerance, as demonstrated by its documented use distinguishing relative blight susceptibility in different species of *Castanea* (Newhouse *et al.*, 2014a).

Leaves from various transgenic lines with the p35S-OxO vector construct have displayed a range of susceptibility (Zhang, B. *et al.*, 2013), but the majority of events (including Darling 58) show similar or higher blight tolerance (i.e. similar or smaller lesions) than naturally blight-tolerant Chinese chestnut controls (Figure 8.1.1a).



Figure 8.1.1a. Representative leaf inoculation lesions (necrotic areas) on three leaf types: non-transgenic (NT) American chestnut, Chinese chestnut, and Darling 58 transgenic American chestnut. Leaves were trimmed after the necrosis formed to facilitate scanning. All images were scanned at 300 dots per inch and cropped equivalently to maintain equal scales.

Leaves of selected Darling 58 T2 seedling offspring (Section 6.4) were compared in a leaf inoculation assay before these trees were planted outdoors in 2019. Leaves were collected and inoculated from ten trees that had inherited the OxO transgene, and ten non-transgenic full-sibling controls. After 6 days, mean necrotic areas were calculated from scanned images and compared via two-sample t-test ($\alpha = 0.05$). Mean necrotic area was significantly smaller ($p = 1.5 \times 10^{-7}$) on leaves that had inherited the OxO transgene (6.1 mm^2) compared to leaves from full-sibling trees that had not inherited the transgene (147.9 mm^2 , Figure 8.1.1b).

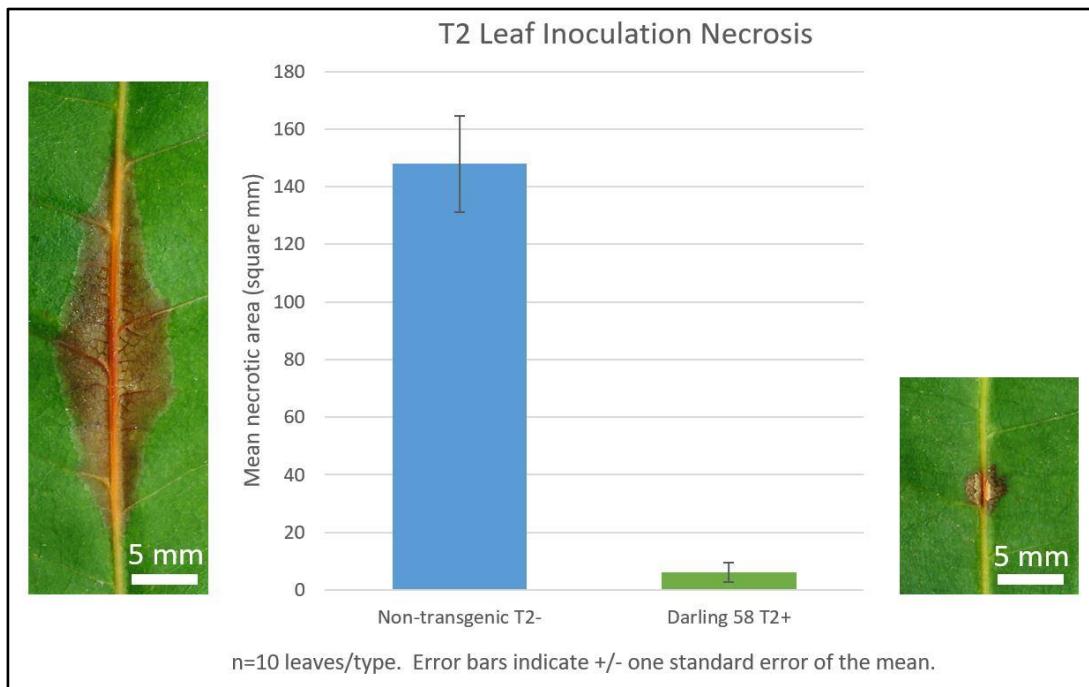


Figure 8.1.1b. Leaf inoculation results from selected T2 offspring of Darling 58. T2- did not inherit OxO; T2+ did inherit OxO. Inset photos show representative leaves with necrotic areas close to the mean of each type (T2- at left, T2+ with OxO at right).

8.1.2 Greenhouse small stem inoculation

Modified small stem blight inoculation assays (Powell *et al.*, 2007) have been performed on stems as small as ~3 – 6 mm diameter. All inoculations in the following small stem assay experiments

were done with *C. parasitica* strain EP155 (ATCC38755), which is a known highly virulent strain regularly used for blight screening (e.g. Cipollini *et al.*, 2017).

In the summer of 2016, we inoculated 12 stems each of Darling 58, Ellis (Non-transgenic American chestnut control), and Qing (Chinese chestnut). This experiment also included transgenic event Darling 54 (Section 10.5.1). Four different researchers inoculated three stems each of each tree type, to avoid bias due to inoculation inconsistencies. Observations consisted of whole tree wilt status, and the experiment concluded when all 12 of the Ellis control trees had completely wilted (61 days post inoculation). At that time point, 9 of 12 Darling 58 trees were still healthy (unwilted). None of the Chinese chestnut trees wilted during the course of this experiment. Despite wilting observed on 25% of the Darling 58 trees (which may have been due to non-blight-related factors such as overwatering during the experiment), the final conclusion is clear: transgenic Darling 58 American chestnuts tolerate blight much better than non-transgenic American chestnut controls.

8.1.3 T1 offspring stem inoculations

Heritability of OxO-based blight tolerance was previously described on older transgenic chestnut events (Newhouse *et al.*, 2014b; Section 10.3). This section describes stem inoculation experiments done on T1 offspring of Darling 58 (Section 6.4).

Blight inoculations on field-growing Darling 58 T1 seedlings (two-season-old trees, Section 6.4) were performed late in the summer of 2018. This included three trees each of transgenic Darling 58 offspring, and non-transgenic full-sibling controls. These inoculations were intentionally aggressive, as they were performed with a relatively heavy inoculum load (larger agar plug) of a highly virulent *C. parasitica* strain (EP155). Despite these severe conditions, the Darling 58 T1 trees showed significantly smaller cankers than their non-transgenic siblings at 18 and 30 days post inoculation ($p < 0.001$), and significantly slower canker progression over time (Figure 8.1.3a). Representative photographs of the cankers at 30 days post inoculation are shown in the same figure; note side view of fully girdled non-transgenic stem. As discussed previously, Darling 58 trees do not kill or repel the blight pathogen, as seen in the visibly infected Darling 58 stem in Figure 8.1.3a, but damage due to blight infection is reduced. All three non-transgenic controls were girdled by the end of the 2018 growing season, while all three transgenic trees had swelling cankers but were not girdled. The small number of seedlings available for this inoculation limits statistical power, but still demonstrates enhanced blight tolerance in Darling 58 offspring.

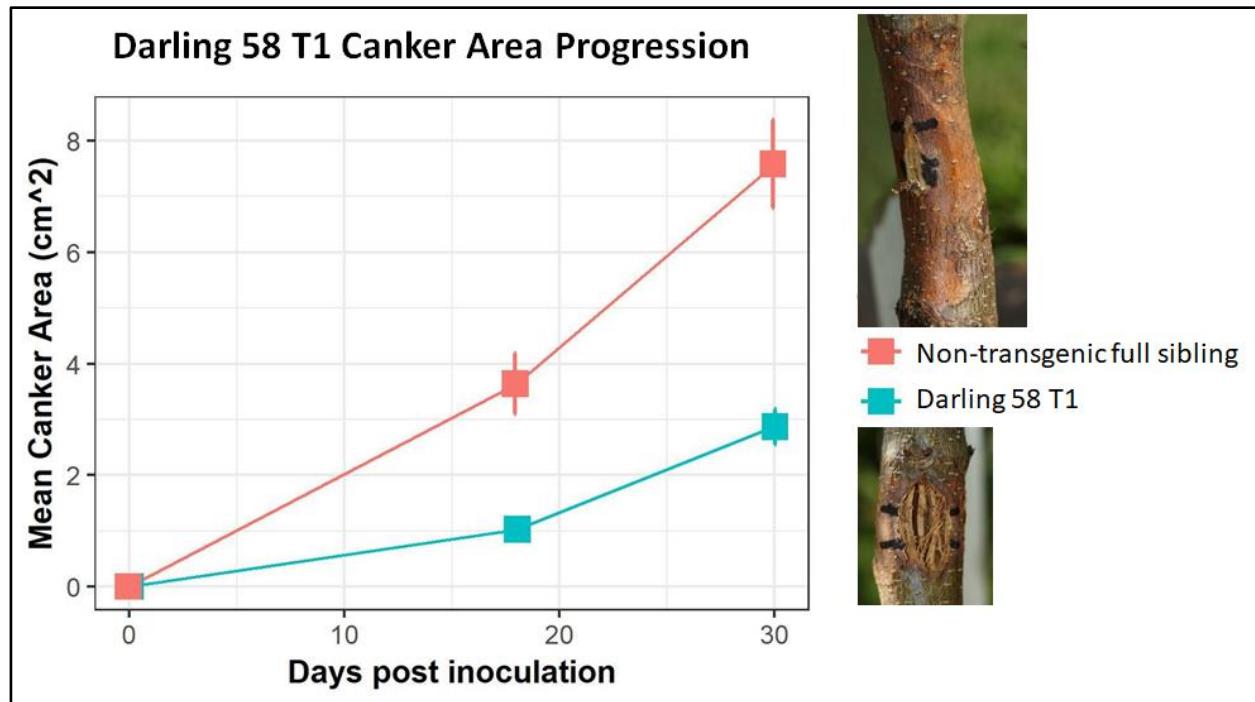


Figure 8.1.3a. Canker area over time following severe stem inoculations on transgenic and non-transgenic Darling 58 offspring. $n = 3$ trees per type, error bars indicate ± 1 standard error of the mean (SEM). Photographs are representative examples of each type, with relative scale retained (black marks show 5 mm initial wound length).

Inoculations were also performed on potted plants that had been vegetatively propagated from Darling 58 T1 seedlings, along with potted non-transgenic American and Chinese chestnut controls. Propagated transgenic T1 lines included D58+16001 (labeled “+001” in Figure 8.1.3b; $n = 40$; the ortet source tree was also inoculated in seedling experiment above) and D58+16020 (“+020”; $n = 10$). Controls consisted of unrelated American chestnut seedlings ($n = 20$) and Chinese chestnut seedlings ($n = 10$). Inoculations were performed by creating a 5mm wound parallel to the stem with a scalpel, applying a 3 mm diameter plug of *C. parasitica* strain EP155, and covering the plug with parafilm for 1 week. All plants were maintained in shade tents (outdoors; inside permitted field plots) for the duration of this experiment. Observations were concluded when all of the surviving infected non-transgenic American chestnut stems were completely girdled by the canker, which occurred 29 days after inoculation. Canker heights were measured parallel to the stem, and means were compared to the non-transgenic American control with 2-sample t-tests ($\alpha = 0.05$). Stems without visible signs of infection ($n = 18$ across all types), and those that died of other causes before girdling ($n = 2$ across all types), were removed from the analysis; see Figures 8.1.3b and 8.1.3c for final numbers of each type.

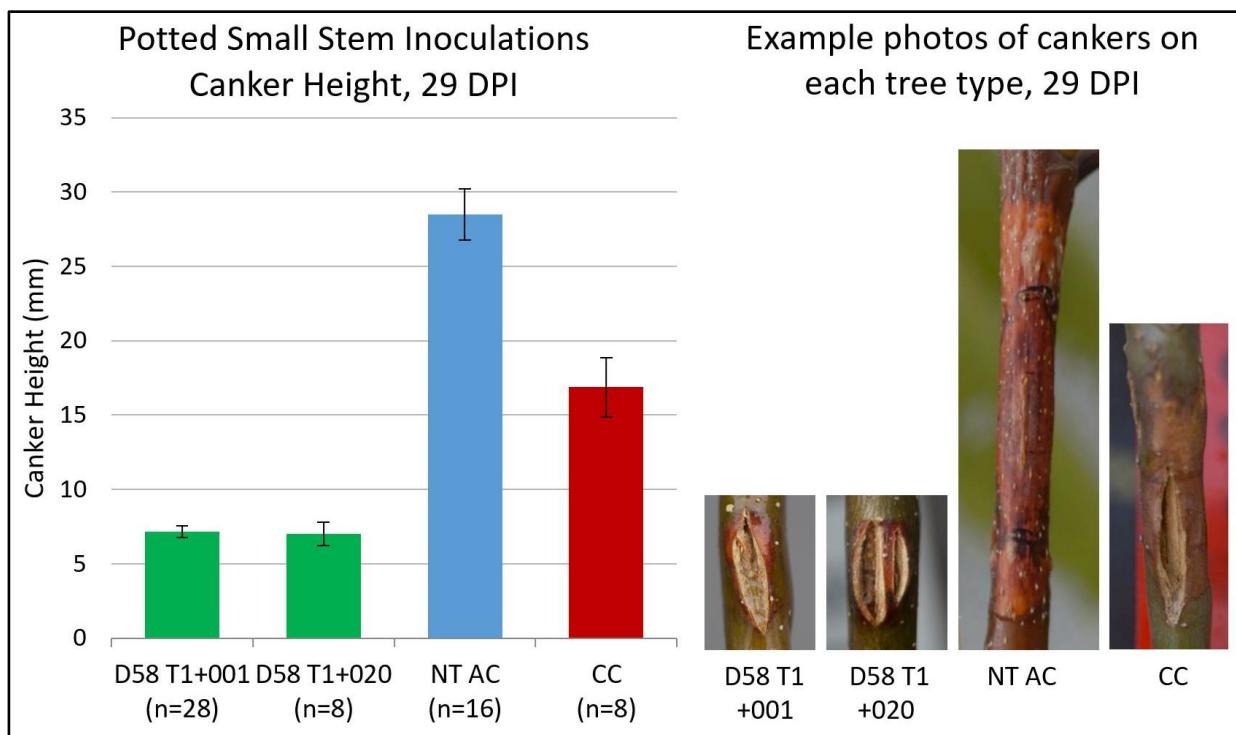


Figure 8.1.3b. Mean canker height (left) on stem inoculations of potted Darling 58 T1 trees compared to non-transgenic controls, with example photos (right) of cankers on each tree type, at 29 days post-inoculation (DPI). Error bars indicate ± 1 standard error of the mean.

Canker height means were compared using ANOVA, and pairwise comparisons were made using Tukey HSD. All analyses were conducted in R (R Core Team, 2019). Canker heights were significantly smaller ($p < 0.001$) on both lines of Darling 58 T1 trees than they were on either Chinese or American control trees. Canker height on Chinese chestnut was also significantly smaller than that on American chestnut ($p < 0.001$) (Figure 8.1.3b).

Darling 58 transgenic stems were visibly infected by blight (Figure 8.1.3b; orange color immediately surrounding the wound), but the infection did not spread far beyond the wound or cause extensive damage like that on the non-transgenic controls. The wounds on the Darling 58 and Chinese stems appear to be splitting open due to ongoing growth in response to wounding and fungal infection, while the non-transgenic American stem tissue surrounding the wound is completely dead due to blight, leaving the wound closed.

Girdling on these inoculated T1 potted plants was categorically evaluated as: not girdled, $>$ half of circumference girdled, or fully girdled. “Not girdled” indicates that less than half the stem circumference was visibly infected, i.e. the canker was not visible when viewed from the back of the stem opposite the inoculation point. “ $> 1/2$ girdled” indicates that greater than half of the stem circumference was infected, but the canker did not completely encircle the stem. “Fully girdled” indicates the canker had completely encircled the stem, which typically leads to death of the stem above the canker. All of the non-transgenic American chestnut stems had been completely girdled by 29 days after the inoculation, while none of the cankers on Darling 58 T1 trees had grown to even 50% of the stem circumference (Figure 8.1.3c). All Chinese chestnut stems were at least half girdled, but only three of the eight Chinese stems were completely

girdled, suggesting a functional level of blight tolerance intermediate between non-transgenic American and Darling 58 chestnuts.

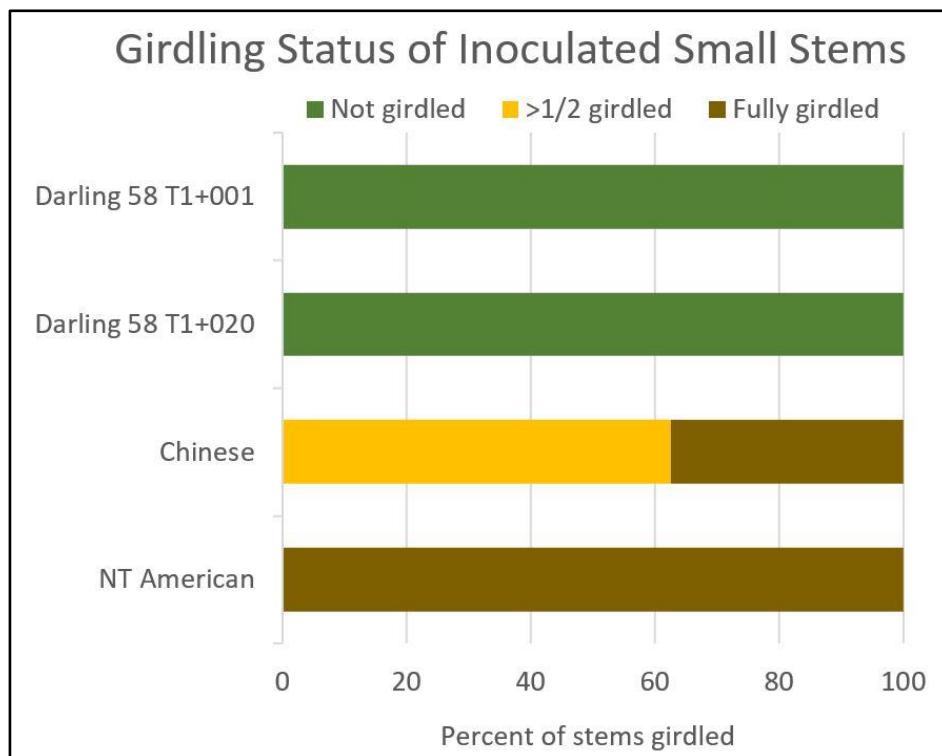


Figure 8.1.3c. Girdling status (percent of trees in each girdling category) 29 days after potted small stem inoculations. Darling 58 T1+001 n = 28, T1+020 n = 8, NT (non-transgenic) American chestnut n = 16, Chinese chestnut n = 8.

8.2 Growth

Over 13 years of anecdotal, unpublished observations indicate that transgenic American chestnuts do not feature unusual or dramatically different growth characteristics compared to non-transgenic controls produced in the same manner. These observations include transgenic and non-transgenic material grown in conditions ranging from tissue culture through growth chambers and greenhouses to field plots (Appendix I). Field release sites have encompassed a variety of environmental site conditions such as isolated open areas with few other trees, controlled experimental plots, tightly spaced holding plots, and shelterwood plots with partial overstories of mature trees. Soils at these plots have ranged from nearly clean sand to organically rich forest humus. Regardless of environmental conditions or cultural treatments, no unusual growth characteristics have been consistently observed due to the presence of the OxO transgene, even in transgenic events containing multiple copies of the transgene or additional reporter genes. There are often easily distinguishable growth differences between trees grown from seed and trees generated from tissue culture, but within either of those sources, we have not been able to consistently visually distinguish transgenic from non-transgenic trees. Appendix I includes APHIS permits and notifications held by ESF and several other institutions for growing or transporting Darling 58 chestnuts; none of these authorizations has included reports of unusual occurrences or deleterious effects due to the transgene.

Due to limitations on numbers of available plants, growth rate of tissue culture-generated plants, and the size of field plots, quantitative measurements comparing growth rates and photosynthetic performance of transgenic vs. non-transgenic American chestnut trees have been limited. The most recent available measurements (Section 8.2.2) are from Darling 58 seedling offspring germinated spring 2019; this is the first year a large sample size (> 10 transgenic and non-transgenic seedlings) of Darling 58 seedling offspring has been available for measurement. However, first-season measurements of chestnut seedling height should be considered preliminary as they are not necessarily indicative of future growth, and may be more closely correlated to nut weight, family background, cultural treatments, or other factors (Pinchot *et al.*, 2015; Clark *et al.*, 2016).

8.2.1 T1 seedling field growth

Darling 58 T1 seedlings (Section 6.4), germinated spring 2017 and planted in the field early summer 2017, were measured after the first and second growing seasons (late fall 2017 and 2018). Blight dieback on non-transgenic trees due to 2018 inoculations (Section 8.1.3) prevented similar comparisons in 2019. Growth was measured in height (distance from ground along the stem to the highest living bud) and diameter (of main stem measured by digital caliper at 10 cm above ground level). Mean height and diameter of the two tree types (transgenic and non-transgenic) were compared with 2-sample t-tests ($\alpha = 0.05$) for each growing season. Half of the measured trees were transgenic (inherited the transgene, $n = 6$) and half were non-transgenic (full siblings that did not inherit the transgene, $n = 6$). Results are shown in Figure 8.2.1a. No significant difference was found between transgenic T1 and non-transgenic plants in any pairwise comparison ($p = 0.073$ for first season diameter; $p > 0.5$ for all other pairwise comparisons).

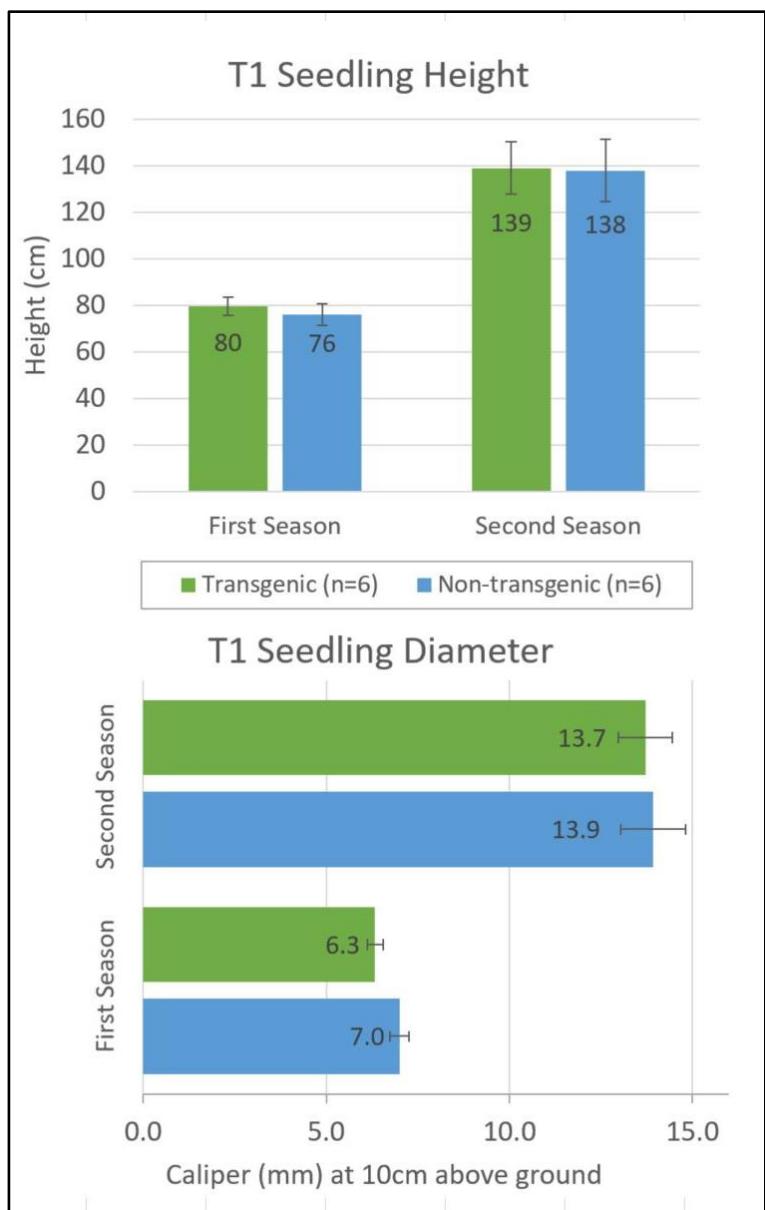


Figure 8.2.1a. Height and diameter of T1 transgenic and non-transgenic offspring of Darling 58 from 2016 pollinations. Error bars indicate ± 1 standard error of the mean.

8.2.2 Early T2 seedling growth

Darling 58 T2 seedlings were germinated in spring 2019, grown in a greenhouse for ~ 2 months, hardened off in a shade tent for ~ 1 month, and planted in early July. Height measurements were recorded in early August 2019 on a subset of these seedlings. These measurements included seedlings from crosses for which there were > 10 surviving transgenic and non-transgenic individuals planted under similar conditions, and excluded interspecific crosses and multiple-stem seedlings. Mean height was analyzed in R (R Core Team, 2019) with a Tukey's HSD test, using pollen type, mother tree, and OxO presence as factors. Figure 8.2.2a shows mean heights of T2 seedlings sorted by both pollen type (i.e. father tree; D58+16001 or D58+16020) and mother tree (two-letter codes along x-axis). The main effects of transgene status (+ or -), pollen type, and

mother tree were significant factors affecting T2 seedling height ($p < 0.05$). In nine out of ten cross types, presence of OxO was not a significant factor affecting tree height. Only one cross type (FA x D58+16001) showed a statistically significant height difference between transgenic (OxO Pos, blue) and non-transgenic (Neg, pink) offspring, with the non-transgenic seedlings from this cross being significantly taller than the transgenic seedlings. Seedlings from some other crosses showed a non-significant trend toward taller non-transgenic trees (e.g. FC x D58+16020), while others showed essentially no difference (e.g. FA x D58+16020). Similar results were observed from earlier height measurements before seedlings were planted outside (not shown), with additional significant differences due to germination date.

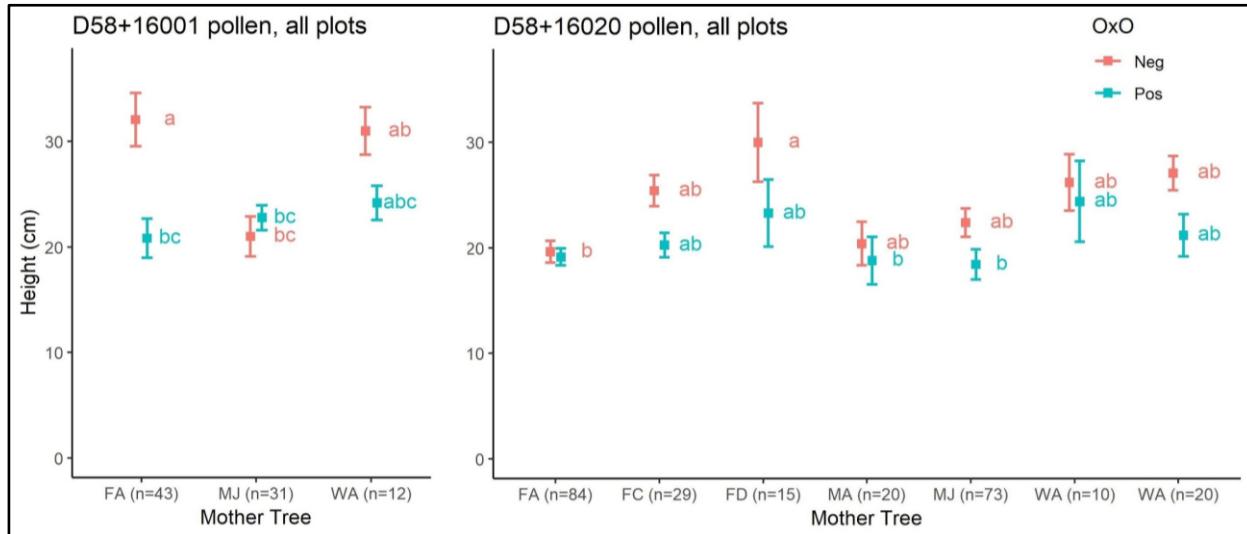


Figure 8.2.2a. Mid-season height growth of Darling 58 T2 seedlings in field plots, grouped by mother tree and T1 pollen type. Error bars indicate +/- one standard error of the mean. Shared letters within a single chart indicate there is not a statistically significant difference between these types, according to Tukey's HSD test ($p > 0.05$).

These preliminary results indicate that presence of OxO in Darling 58 offspring may be correlated with a reduction in growth rate in some crosses during the first few months after germination, and that other factors affect growth to similar or greater extents. However, these plantings were not set up for direct evenly replicated experimental comparisons, and the mid-season timing may reflect other factors such as seedling storage before field planting, planting methods, or field planting date, which were not controlled or evenly replicated in this post-hoc comparison. Therefore, data and conclusions should be considered preliminary until measurements can be conducted on older seedlings in controlled experimental plots. Growth data on older paired T1 trees do not show consistent trends according to transgene presence: in fact, there was essentially no difference between transgenic trees and their non-transgenic full siblings after two years of growth (Section 8.2.1). In order to obtain better long-term growth data, many T2 seedlings (including transgenic and non-transgenic full-sibling pairs) have been planted in experimental plots specifically with future growth comparisons in mind.

Small growth differences with significant parental source effects corroborates previous chestnut growth comparisons, which also report that mother tree source (i.e. family background) of chestnut seedlings significantly affects growth, along with various other factors and conditions

(Bauman *et al.*, 2014; Clark *et al.*, 2016; Thomas-Van Gundy *et al.*, 2017). Early growth (less than one growing season) may reflect seed size or other factors, and may not predict long-term growth rates. For example, studies on backcross chestnuts have shown that first-year height growth is correlated with nut size, planting method, selection technique, or family background, but such differences do not necessarily persist or predict growth beyond the first growing season (Clark *et al.*, 2012; Bauman *et al.*, 2014; Pinchot *et al.*, 2015; Cipollini *et al.*, 2019). One study on chestnut establishment (Clark *et al.*, 2016) specifically describes a phenomenon called “planting shock”, which results in negligible growth during the first year after outplanting, but this effect disappeared by the second growing season. Authors of another chestnut reintroduction study (Griscom and Griscom, 2012) caution against applying greenhouse growth comparisons to field growth conclusions, since these environmental conditions and early growth are not consistent with longer-term field growth. The fact that the measured T2 seedlings had been in the ground for only about a month before height was measured suggests greenhouse or cultural conditions may be a significant factor in growth at this stage, so measurements in subsequent growing seasons (as with the T1 trees) should be more informative.

Other chestnut studies, mostly comparing backcross to full American trees, confirm that growth measurements collected soon after germination may not predict long-term trends. Early growth of Chinese or hybrid trees may be faster than American (Bauman *et al.*, 2014; Cipollini *et al.*, 2019), probably due to the larger nut size, but after one or more full growing seasons, American chestnut tends to grow faster and taller than Chinese or hybrid chestnuts under the same conditions (Clapper, 1954; Clark *et al.*, 2012). Bauman *et al.* (2014) found that backcross trees had a mean growth rate 12 – 15% lower than American after 30 months, and 16 – 17% lower after 60 months (data from table 2 in Bauman *et al.* 2014). Pinchot *et al.* (2015) compared growth of American, Chinese, BC₃F₂ and BC₃F₃ seedlings after one full growing season, and found the mean height of American chestnut seedlings to be greater than either BC₃F₃ or BC₃F₂ seedlings, though only the difference between American and BC₃F₂ (a 17% reduction) was statistically significant. The growth of Chinese chestnut was greater than all other tree types, but the difference between Chinese and American was not statistically significant (data from Table 4 in Pinchot *et al.*, 2015). Rieske *et al.* (2003) found that mean height of F1 (Chinese x American hybrid) seedlings was 14% less than that of American chestnut seedlings after 10 weeks of growth (data from Table 3 in Rieske *et al.*, 2003). These differences give context to the small growth reduction (an 8% mean reduction for all seedlings included in the analysis) reported above in transgenic T2 chestnuts as compared to related non-transgenic T2 chestnut seedlings. Specifically, even if the growth difference observed in some T2 seedlings persists as trees mature, traditional breeding and natural genotypic differences appear to have a greater impact on growth than genetic engineering.

If the growth differences observed in some transgenic seedlings are found to be consistent, this difference could be explained by a metabolic cost of expressing the OxO enzyme (Molla *et al.*, 2013; Karasov *et al.*, 2017; Section 8.3). Potentially reduced first-season growth and increased blight tolerance of both transgenic chestnuts and hybrid chestnuts described in this section fits with the growth-differentiation balance hypothesis, in which production of secondary metabolites for defense diverts resources from production of biomass. This has been observed in other plants (Karasov *et al.*, 2017) and even hybrid chestnuts (Rieske *et al.*, 2003), so if it represents a biologically significant effect in transgenic chestnuts, it would not present novel plant pest risks

compared to traditional breeding. This hypothesis is not supported by respiration and photosynthesis measurements on T2 seedlings, which did not show significant differences according to transgene presence: see Section 8.3 for further discussion of this topic.

Whether it is due to transgene expression or hybrid chestnut parentage, a slight reduction in growth may be an inevitable but acceptable price to pay for blight tolerance, as healthy blight-tolerant chestnuts will ultimately achieve far greater growth than chestnuts that succumb to disease. Preliminary observations comparing OxO expression (measured by RT-qPCR) and early height growth suggest there is not a strong correlation between these factors, but tests will be conducted on older trees and a greater number of individuals before this hypothesis can be conclusively tested. Further research is also ongoing into genetically engineered trees that produce OxO in a more targeted manner (i.e. only in vascular tissue and/or induced as a response to wounding or fungal infection), which could lower the metabolic cost of OxO expression (Karasov *et al.*, 2017).

8.3 Respiration and photosynthesis

One consideration for transgenic plants is whether constitutive transgene expression will affect plant functions such as respiration and photosynthesis. Respiration and photosynthesis are central components of the primary metabolism of plants and strongly influence growth rates (Atkin and Tjoelker, 2003). Photosynthetic rates and the light-use efficiency of photosynthesis are sometimes higher in invasive or weedy plants relative to non-weedy natives (e.g., Heberling and Fridley, 2013; Fan *et al.*, 2013), so increased weediness might be a valid concern if transgenic trees exhibited markedly higher photosynthetic rates relative to non-transgenic controls. Respiration refers to the CO₂ production rate of plant organs resulting from the mitochondrial breakdown of carbohydrates, and thus reflects an integrated measurement of the rate of metabolism (Atkin *et al.*, 2015; Atkin and Tjoelker, 2003; O'Leary *et al.*, 2019). Treatments that increase the activities of plant metabolic pathways also tend to increase the rate of respiratory CO₂ production (Leakey *et al.*, 2009), as respiration produces the adenosine triphosphate (ATP) and C-skeletons that support metabolism (O'Leary *et al.*, 2019). Respiration is most conveniently measured in the dark at night (R_{dark}), when CO₂ production is not confounded with photosynthetic CO₂ assimilation. However, respiratory CO₂ production continues via mitochondrial activity during sunlight periods during the day (R_{light}), and detailed gas-exchange measurements are required for the measurement of R_{light} (Heskel *et al.*, 2013).

Drake *et al.* (Sections 8.3.1 – 8.3.4; Duong and Drake, 2018) measured photosynthetic light-response curves, photosynthetic CO₂ response curves, and dark respiration rates of mature leaves of transgenic American chestnut trees and related controls during the summers of 2018 and 2019. The purpose of these measurements was to assess potential transgene impacts on plant metabolism, particularly the rates of foliar respiration (R_{dark} and R_{light}) and the photosynthetic capacity. The light response of photosynthesis can be well described by four parameters: the maximum net CO₂ assimilation rate at high light (A_{max} or A_{sat}), the slope of the light response of photosynthesis at low light (i.e., the apparent quantum yield, α), the rate of net CO₂ release in the dark (R_{dark}), and a curvature parameter describing the bend between the linear and asymptotic region of the light response curve. Photosynthetic capacity at high light can be measured by CO₂ response curves (i.e., A:C_i curves), which quantify the maximum rubisco

carboxylation rate ($V_{c,\max}$) and the maximum rate of electron transport through the light reactions of photosynthesis (J_{\max}).

The measurements described in Sections 8.3.1 – 8.3.3 were performed in 2018 on 14 Darling 58 offspring (T1 generation) in their third growing season (Section 6.4) planted in the ground at a permitted location in Syracuse, NY. Six of the trees inherited the OxO transgene from the parental transgenic pollen (Darling 58), and eight trees were full siblings that did not inherit the OxO gene. The 2019 measurements (Section 8.3.4) included light-saturated photosynthesis (reported as A_{sat} , rather than light-response curves) and R_{dark} measurements, and were conducted on 70 seedlings including transgenic and non-transgenic Darling 58 T2s and hybrid controls, planted in two locations (NY and PA).

In both the 2018 and 2019 studies, we investigated whether transgenic trees showed different photosynthetic capacity or metabolic costs associated with maintaining leaves, such that respiration rates would change in transgenic trees relative to non-transgenic controls. These studies did not specifically distinguish direct effects of the transgenes (OxO and NPTII) from other factors, such as potential effects of linked endogenous chestnut genes in these crosses. Even though *expression* of nearby endogenous genes is apparently not affected by the presence of the transgenes (Section 7.2.3), any genes that are nearby on the same chromosome as the insert are considered linked, and will therefore be present in transgenic offspring at higher proportions than unlinked genes. Such linkages can be reduced with adequate numbers of crosses or through marker-assisted selection (Westbrook *et al.*, 2019a), but are likely ubiquitous in these small numbers of T1 offspring.

8.3.1 Light response curves

We measured high-resolution photosynthetic light response curves on one fully developed leaf on each tree using a standard portable gas exchange system (Licor 6800, Licor Biosciences, Lincoln, NE, USA). We chose fully expanded leaves of similar size and age in the top third of the canopy for measurement. We set the initial leaf cuvette conditions at the ambient CO_2 concentration of $400 \mu\text{mol mol}^{-1}$, a saturating light intensity of $1800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, and a vapor-pressure deficit of $1 - 1.5 \text{ kPa}$ at a leaf temperature of 25°C . We then recorded net rates of photosynthesis at light intensities of $1800, 1800, 1500, 1000, 500, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 0, 0$, and $0 \mu\text{mol m}^{-2} \text{s}^{-1}$. The repeated measurements at 1800 and $0 \mu\text{mol m}^{-2} \text{s}^{-1}$ were used to ensure stability of leaf gas exchange. We used the Kok method to estimate mitochondrial respiration rates in the light (R_{light}) as the y-intercept of a linear regression between net photosynthesis and incident light at light levels between 30 and $90 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Villar *et al.*, 1994; Heskel *et al.*, 2013). We used the final measurement at a light intensity of $0 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ as an estimate of mitochondrial respiration in the dark (R_{dark}). We also fit non-rectangular hyperbolas (Marshall and Biscoe, 1980; Cannell and Thornley, 1998) to all of the individual photosynthetic light-response curves to estimate additional photosynthetic parameters including: the maximum rate of photosynthesis under saturating light (A_{\max}), the apparent quantum yield (α), and the curvature parameter (θ), the light compensation point (LCP; the light level at which net photosynthesis was zero), and the light saturation point (LSP; the light level at which photosynthesis was 90% of its maximum value). Thus, R_{light} , R_{dark} , and the other parameters were estimated by standard and common gas exchange techniques in the field (Heskel *et al.*, 2013;

Cannell and Thornley, 1998). We used analysis of variance (ANOVA) to determine possible statistical differences between transgenic and non-transgenic tree groups. The residuals of the ANOVAs were examined and satisfied the assumptions of homoscedasticity.

The results of these light-response curve measurements are shown in Figure 8.3.1a, and the statistical analysis of the parameters that were extracted from these curves are shown in Table 8.3.1a. We found that the light response curves were not significantly different between T1 transgenic and non-transgenic trees (Figure 8.3.1a, panel a) and rates of R_{light} and R_{dark} were similar as well (Figure 8.3.1a, panel b). We found that foliar respiration was suppressed by ~25% by light (i.e., R_{light} was less than R_{dark}), but this effect was equivalent across transgenic and non-transgenic trees. There was a trend towards higher light-saturated photosynthetic rates in transgenic trees, but this difference was not statistically significant (Table 8.3.1a), and both tree types were within the range observed on non-transgenic chestnuts in a previous study (Knapp *et al.*, 2014). We recognize the modest replication of the measurements here. Both transgenic and non-transgenic trees had light compensation and light saturation points of 25 and 700 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively, which are relatively low values as would be expected for shade-tolerant forest trees (Craine and Reich, 2005; Kubiske and Pregitzer, 1996). These results suggest that the transgene insertion did not affect the respiratory or photosynthetic physiology of American chestnuts growing in this orchard setting.

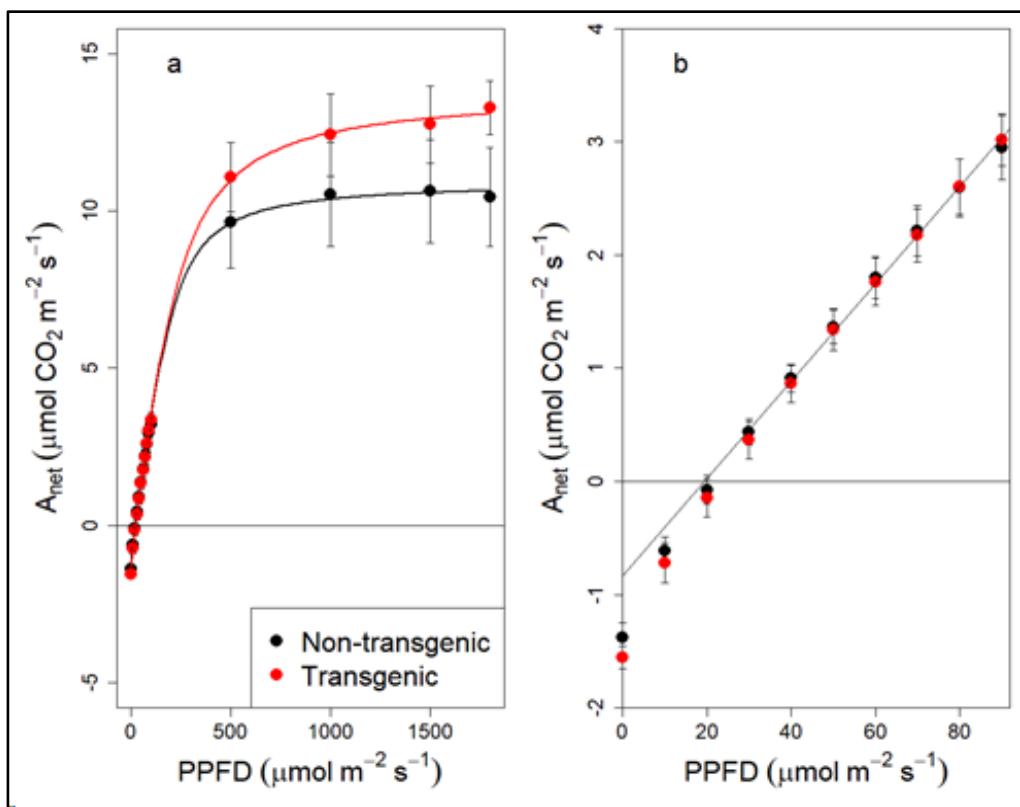


Figure 8.3.1a. Photosynthetic light response curves across a wide range of light levels (left/a), and at low light levels only (right/b). The measured net rate of leaf photosynthesis (A_{net}) was recorded at a range of light levels (photosynthetic photon flux densities: PPFD). Each symbol reflects the mean of 6 – 8 leaves per treatment and the error bars reflect 1 SEM. At right (b), the respiration rate in the light (R_{light}) was estimated as the y-intercept of a linear model fit to the data from 30 – 90 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and the respiration rate in the dark was measured at a PPFD of 0 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. There were no statistically significant differences between transgenic and non-transgenic trees.

Table 8.3.1a. Mean light response variables across all tree types. Statistical analyses of photosynthetic light response parameters indicate no significant difference between transgenic and non-transgenic trees ($\alpha = 0.05$), so the means presented here reflect both groups. Most of these parameters were estimated by fitting a non-rectangular hyperbola to the entire dataset (see body of Section 8.3.1), and thus include data from all light levels.

| Variable | Mean value | F-value | P-value |
|--------------------|--|---------|---------|
| R_{light} | -0.8 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ | 1.5 | 0.2 |
| R_{dark} | -1.2 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ | 0.3 | 0.6 |
| A_{\max} | 13.2 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ | 1.1 | 0.3 |
| α | 0.055 $\mu\text{mol CO}_2 \mu\text{mol photons}^{-1}$ | 0.2 | 0.7 |
| θ | 0.77 | 0.0 | 0.9 |
| LCP | 25.1 $\mu\text{mol photons}^{-1} \text{ m}^{-2} \text{ s}^{-1}$ | 1.1 | 0.3 |
| LSP | 700.7 $\mu\text{mol photons}^{-1} \text{ m}^{-2} \text{ s}^{-1}$ | 0.0 | 0.9 |

We performed these high-resolution light response curves at two timepoints during the summer of 2018 with the primary purpose of measuring R_{light} . There was a modest and non-significant trend towards a higher rate of R_{light} in the transgenic trees at the first timepoint (June), but there was no difference between transgenic and non-transgenic trees at the second timepoint (July; Figure 8.3.1b).

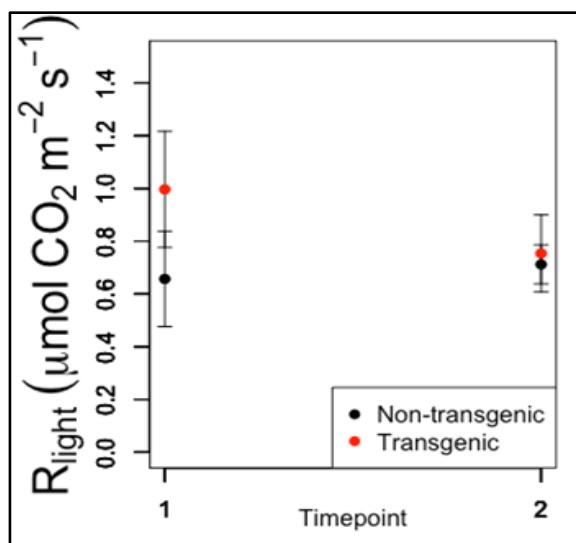


Figure 8.3.1b. Rate of mitochondrial respiration in the light (R_{light}) for transgenic and non-transgenic trees measured in June (timepoint 1) and July (timepoint 2) using high-resolution light response curves. Values reflect the mean of 6 – 8 trees per treatment, and the error bar reflects the standard error (SE). There was no significant difference in R_{light} between treatments.

8.3.2 Photosynthetic CO₂ response curves

Given the non-significant but clear trend towards higher light-saturated photosynthetic rates in T1 transgenic trees (Figure 8.3.1a), we performed additional measurements to quantify the photosynthetic capacity of transgenic and non-transgenic trees. We performed CO₂-response curves and extracted parameters describing the maximum capacity of rubisco carboxylation ($V_{c,max}$) and the maximum rate of electron transport through the light reactions of photosynthesis (J_{max}) using standard leaf-level gas exchange methodology (Duursma 2015). We performed these measurements on the same set of T1 trees (6 transgenic Darling 58 T1s and 8 non-transgenic control trees) in September 2018. The $V_{c,max}$ results demonstrate a lower maximum rate of RuBisCO carboxylation for transgenic plants than for non-transgenic plants. The J_{max} results demonstrate a lower maximum rate of electron transport through Photosystems I and II for transgenic plants as well. These results suggest that the photosynthetic capacity of transgenic trees may be modestly lower than the photosynthetic capacity of non-transgenic control trees (Figure 8.3.2a). If this effect is biologically significant, it would result in slower growth rates on the trees with lower photosynthetic capacity, which was not observed on the T1 seedlings in this test (Figure 8.2.1a).

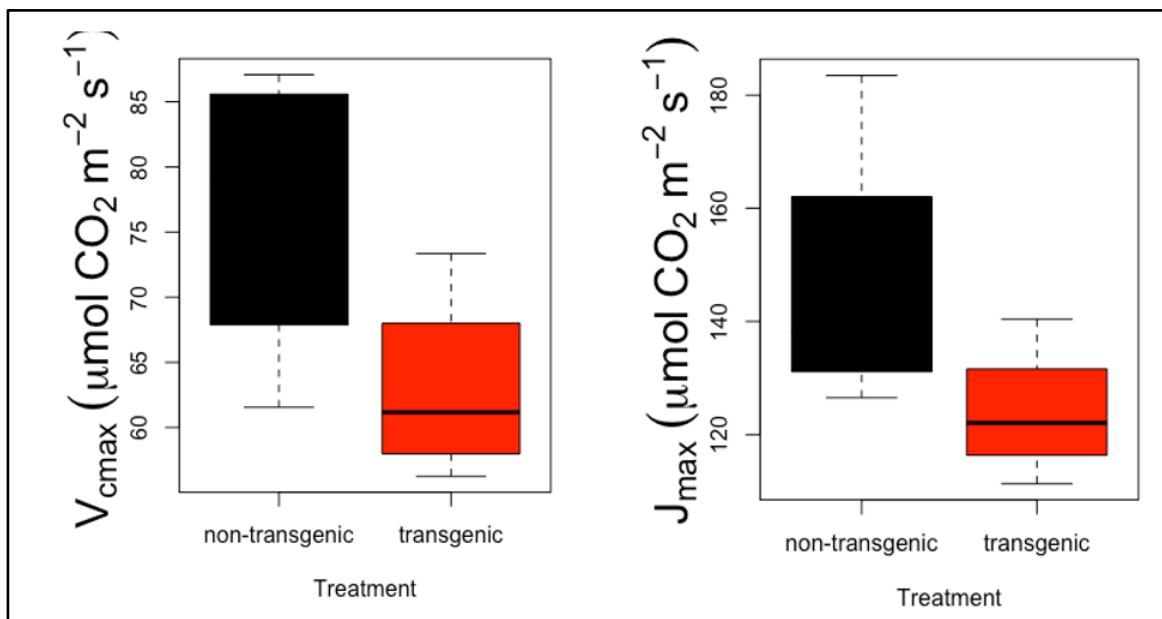


Figure 8.3.2a. Photosynthetic capacity parameters $V_{c,max}$ (left) and J_{max} (right) for transgenic and non-transgenic photosynthetic response CO₂ response ($A-C_i$ curves).

8.3.3 Leaf dark respiration

We also measured the rate of mitochondrial respiration in the dark of mature leaves of these trees. These measurements were performed at night in June, July, and September of 2018 on six Darling 58 T1 trees and eight non-transgenic control trees.

In June and July (timepoints 1 and 2), leaf R_{dark} was significantly higher (+25%) in transgenic trees relative to non-transgenic trees (Figure 8.3.3a). However, R_{dark} was equivalent across treatments in September (Figure 8.3.3a). These results suggest that there may be a small metabolic cost of

constitutive transgene expression that requires an increased metabolic rate (i.e., enhanced ATP production to support synthesis of the transgene product). This effect has the potential to reduce the growth rate of transgenic trees, although no growth differences were detectable on these same T2 trees after two growing seasons (Section 8.2).

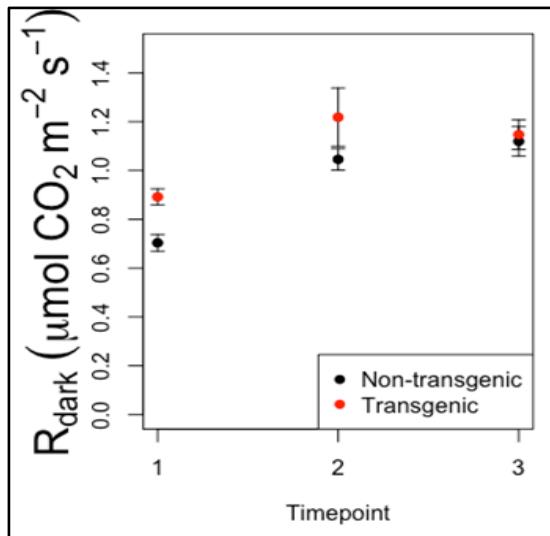


Figure 8.3.3a. Mean mitochondrial leaf respiration rates measured in the dark (at night) for 6 transgenic and 8 non-transgenic trees at three timepoints (June, July, and September 2018). Error bars indicate +/- 1 standard error of the mean.

8.3.4 Photosynthesis of T2 seedlings

Measurements were conducted on a different set of chestnuts in the summer of 2019. Saturated photosynthesis (A_{sat}) and dark respiration (R_{dark}) were calculated from 11 to 12 replicate measurements on three tree types located in two different sites (NY and PA). Tree types included transgenic and non-transgenic T2 offspring of MJ mother trees (Section 6.4), and ‘Hampchuria’ (a complex hybrid including Korean and American chestnut). The two sites were planted and measured at slightly different times and under different conditions, so site-to-site comparisons were not directly analyzed. Instead of measuring R_{dark} at night, individual leaves were wrapped in foil for > 2 hours prior to respiration measurements, and all measurements were conducted during the day. For each measurement (R_{dark} and A_{sat}), mean respiration and photosynthesis in each plant type were compared with Tukey’s HSD tests using the agricolae package in R (de Mendiburu, 2019; R Core Team, 2019). Contrasting A_{sat} trends at the two sites suggests that planting location, environmental conditions, or other external factors are more important factors than tree type (Figure 8.3.4a). R_{dark} measurements do not show differences between tree types at either site, but again suggest that site effects may be more important than genotype effects.

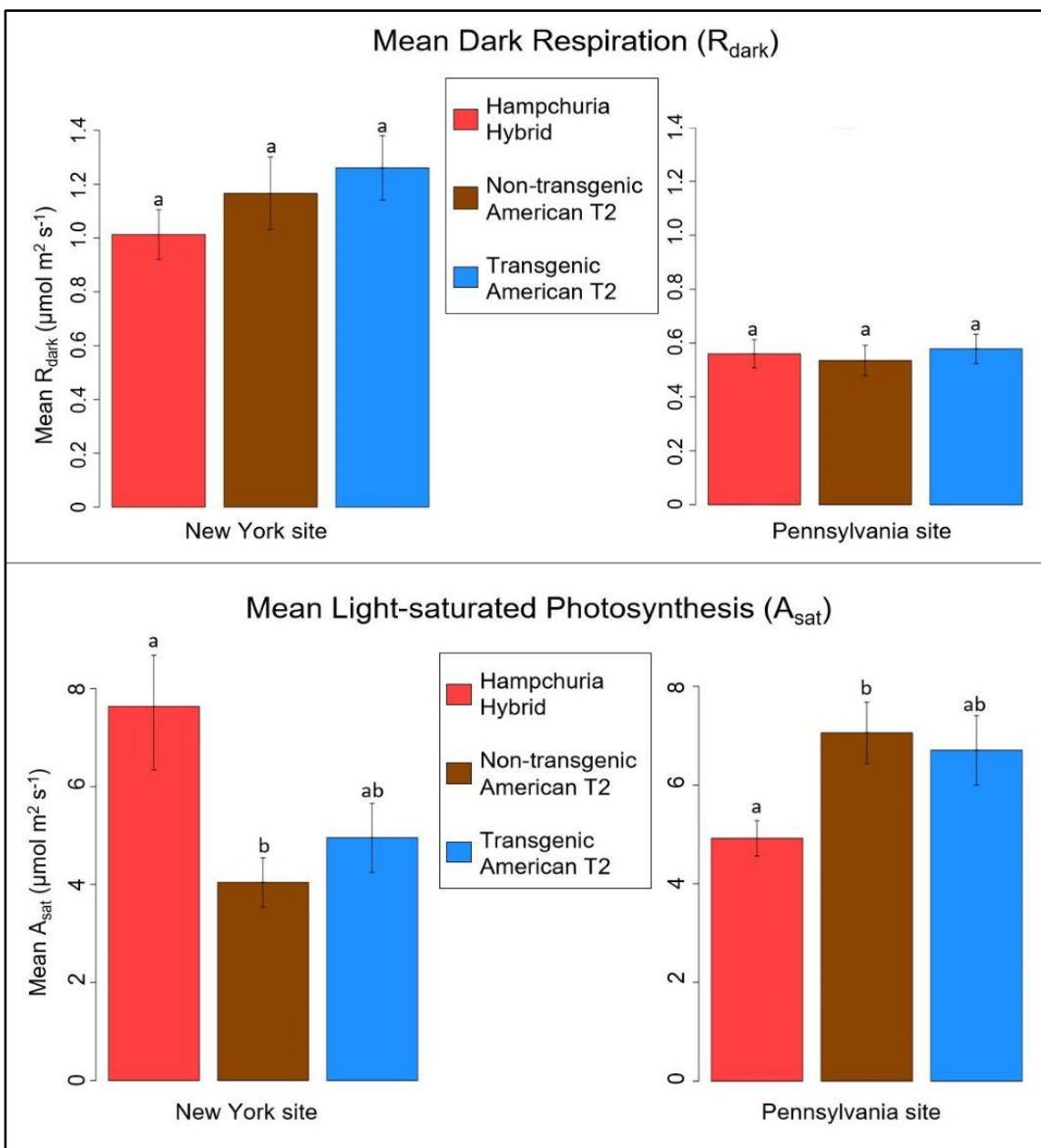


Figure 8.3.4a. R_{dark} and A_{sat} by tree type and site. Error bars indicate \pm one standard error of the mean. Shared letters within each site indicate non-significant differences according to a Tukey's HSD test ($p > 0.05$). $n = 12$ seedlings per type at each site, except Transgenic T2 and Hampchuria in Pennsylvania, for which $n = 11$.

8.3.5 Summary of photosynthesis and respiration results

Collectively, we found that the transgenic trees had similar photosynthetic and respiratory rates relative to non-transgenic control trees. We did find some correlations between transgene presence and an increased rate of respiratory release of CO₂ during some timepoints on T1 trees (Figure 8.3.3a), and correlations between transgene presence and reduced photosynthetic capacity on T1 trees (Figure 8.3.2a), but it is possible that these differences are due to endogenously linked chestnut genes instead of the transgenes themselves. These results were

not consistent with measurements on T2 seedlings, which showed opposing trends at different planting locations (Figure 8.3.4a). These correlations between photosynthetic activity and transgene presence in T1 trees, while not statistically significant in the current studies, could hypothetically reduce the growth rate of transgenic trees relative to non-transgenic control trees by reducing carbon (C) uptake and increasing C release back to the atmosphere. If these effects are biologically significant, they apparently have a small or negligible impact on tree growth rates, given that height and diameter of the tested T1 trees were not significantly different after two growing seasons (Figure 8.2.1a).

Mid-season height differences in certain families of T2 transgenic seedlings (Section 8.2.2) could be explained by this differential carbon uptake and release, but according to photosynthesis and respiration measurements performed on one family of T2 seedlings, photosynthetic activity does not differ according to transgene presence. And as with the T1 trees, T2 height differences were small and not consistent among all families. If these effects noted on T1 trees are biologically significant and persist or grow as Darling 58 offspring get older, a modestly lower photosynthetic capacity (and thus potentially slightly reduced growth rates) in transgenic plants would suggest that Darling 58 offspring could actually be less weedy or aggressive than otherwise-similar wild-type American chestnut. However, photosynthetic activity of T2 seedlings and hybrids at different sites suggests that planting location, environmental factors, or hybridization have larger impacts on photosynthesis than transgene presence. For comparison, a separate study on chestnut photosynthesis reported similar photosynthetic rates for American and backcross chestnuts, but significant differences between these types and Chinese chestnut (Knapp *et al.*, 2014). Other experiments involving chestnut photosynthesis have shown differences among chestnuts grown under different thinning regimes (Joesting *et al.*, 2007), and differences among chestnuts of different age (Joesting *et al.*, 2009).

We recognize that these analyses reflect a small number of measurements on a limited number of trees, and that they do not include other non-transgenic American chestnut types that would help put the results in context of natural variation. We also recognize that there could be biological effects of transgene insertion or expression on the photosynthetic and respiratory rates that we were unable to detect here, that such biological effects would only manifest at particular times of year or in particular growth conditions, that any of these effects might be due to linked endogenous chestnut genes near the insertion site rather than the insertion itself, or that such effects may be smaller than those caused by traditional breeding or other treatments. Finally, we have an ongoing effort to more fully characterize the photosynthetic and respiratory physiology of these trees (and others) in three common gardens across a climate gradient that will progress over the next few years (see BRAG project description, Section 11.2); results will be published and/or shared as they become available.

8.4 Nut nutrition and composition

We do not anticipate pure American chestnuts becoming a prominent agricultural product, regardless of blight tolerance or transgene status, as other *Castanea* species and hybrids appear better suited to nut production and harvest (due to larger nut size, easier peeling characteristics, smaller orchard-friendly tree form, etc.). But if, or when, healthy American chestnuts are able to mature, flower, and produce nuts in the wild, it is almost certain that the nuts will be readily

consumed by both people and wildlife. This will be the case regardless of the method used to produce these potential restoration materials (Section 3.3). In addition to nutritional quality for people and animals, and other potential health effects discussed in this section, nutrition and tannin analyses provide information about the composition of transgenic nuts compared to their non-transgenic counterparts, which is directly relevant to broader environmental interactions. A discussion of the lack of association between OxO and gluten, though less relevant to environmental interactions, is a concern for many people and is presented in Section 11.5.

It is also worth noting that in chestnuts the nut “meat” is primarily cotyledon tissue, which is derived from a fertilized cross between two parent trees, and as a result the pollen source can significantly affect nut characteristics (Anagnostakis, 2009). This is different from other food products like apples, in which the consumed portion of the fruit is derived from maternal flower tissue, and pollen source is not expected to affect fruit characteristics.

8.4.1 Nutrition analysis

In order to compare nutrition and macronutrient composition information of transgenic and non-transgenic American chestnuts, nuts from Darling 58 pollinations of non-transgenic mother trees were submitted for testing by a commercial nutrition testing lab on two separate occasions. Nutritional analyses on Darling 58 nuts were completed by Medallion Labs (Minneapolis, MN) in 2017 and 2018; full reports from the testing lab are available in Appendix VI, and summary data from both analyses are shown in Table 8.4.1a. Data in this summary chart have been rounded to FDA specifications for nutrition labels, which simplifies broad-scale comparisons and puts numbers in a context of nutritional significance. Additional analyses were previously completed on Darling 4 T1 nuts and other non-transgenic control chestnuts; see Section 10.5.2 and Appendix VII for details. Summary data from non-transgenic controls harvested concurrently with Darling 4 T1 nuts in 2016 are shown in Table 8.4.1a for ease of comparison.

Prior to testing, transgenic nuts were identified by removing a small nut core (Section 7.4.2), and nuts that tested positive for OxO presence according to a histochemical assay (Figure 7.4.1b) were separated from nuts that didn’t inherit the transgene. Each nutrition analysis sample consisted of a pool of approximately 20 nuts from a single cross (one pollen type on one mother tree). Therefore transgenic and non-transgenic samples in each analysis are comprised of full siblings from the same pollinations. Nuts from other American chestnut trees in nearby plots, and nutritional data from other frequently consumed *Castanea* species, are included for comparison.

Previous analyses of macronutrient content in American chestnuts specifically are extremely limited, but one study (Anagnostakis, 2009) reported nutrient levels in one cross of American chestnuts. This study generally reports similar values to American chestnuts in our tests. When converted to the 50g serving sizes shown in our table, nutrient levels reported by Anagnostakis are: ~2.2 g protein, ~3.5 g fat, and ~20 g carbohydrates.

As seen in Table 8.4.1a, full-sibling transgenic and non-transgenic nuts from a given year are nutritionally almost identical when rounded to FDA guidelines for food labels: any differences are smaller than those found between *Castanea* species or between other non-transgenic American chestnut trees. American chestnut nutritional measurements do vary between source trees, and between similar trees over different years (as noted for other species below), but transgenic

chestnuts do not fall outside any ranges found in our analyses of non-transgenic chestnuts. Therefore the presence of the OxO transgene or the transformation process do not cause nutritional differences beyond those already present in traditionally bred chestnuts. This is similar to other OxO-transformed crops which have been analyzed for various aspects of nutritional quality: Hu *et al.* (2014) reported that OxO-expressing peanut lines were similar to non-transgenic parent lines in terms of mineral nutrients, fatty acids, composition, fiber, and grade (quality), while Donaldson *et al.* (2001) and Cober *et al.* (2003) both reported no differences in seed protein or oil content of OxO-transformed soy. Our nutrition evaluations also provide macronutrient information indicating that Darling 58 transgenic chestnuts are not compositionally different than non-transgenic chestnuts, and so the nuts should not present novel nutritional risks to consumers or unique risks to the environment based on macronutrient composition.

Previous analyses on several chestnut species have shown that significant nutritional differences can be found between nuts from different tree varieties, or from the same varieties grown on different sites (McCarthy and Meredith, 1988; Borges *et al.*, 2008; Anagnostakis, 2009; de Vasconcelos *et al.*, 2010b; Neri *et al.*, 2010; Yang *et al.*, 2015). This is also demonstrated in our data below, in which there are differences among source trees or sample years. Additionally, this variation suggests that the samples in Table 8.4.1a should be considered examples, not averages representative of entire species.

Table 8.4.1a. Chestnut nutrition and composition information for Darling 58 and controls.

This table summarizes nutrition data for chestnuts including T1 and T2 Darling 58 transgenic American (shaded green), full-sibling non-transgenic (NT) American controls, four unrelated non-transgenic American chestnuts (NT AC...x OP), one NT American chestnut x B3F3 (backcross chestnut parent; Section 3.3), one non-transgenic offspring of a cross with Darling 4 (Dar4-NT), and two other Castanea species (shaded yellow). All values have been rounded to FDA specifications for presentation on nutrition labels in order to enhance clarity; original values are found in Appendix VI (2017 and 2018 analyses, Darling 58 and controls) and Appendix VII (2016 analyses). Note that Vitamin C and iron values as measured in 2017 for both sample types were lower than most measured in other years; see Appendix VI for Medallion Labs correspondence confirming a lack of instrumentation error for these tests.

| Chestnut type: | Darling 58 T2+ | Full Sibling of Darling 58 (T2-NT) | Darling 58 T1+ | Full Sibling of Darling 58 (T1-NT) | NT AC (Clark) x OP | NT AC (Wisconsin) x OP | NT AC (Moss Lake) x OP | NT AC (Zoar) x OP | NT AC (McCabe) x B3F3 | NT AC (McCabe) x Dar4-NT | European (purchased retail) | Chinese (database values) |
|-------------------------|-------------------|--|-------------------|--|--------------------------|------------------------------|------------------------------|-------------------------|-----------------------------|--------------------------------|-----------------------------------|---------------------------------|
| Serving Size: | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~5 nuts) | 50g (~5 nuts) |
| Calories | 120 | 120 | 100 | 100 | 110 | 120 | 80 | 110 | 120 | 120 | 90 | 110 |
| Cal. from fat | 20 | 30 | 20 | 20 | 20 | 30 | 10 | 20 | 40 | 40 | 10 | 10 |
| Total fat (g) | 2.5 | 3.5 | 2.5 | 2.5 | 2.0 | 3.5 | 1.5 | 2.0 | 5.0 | 4.5 | 0.5 | 1.5 |
| Sat. fat (g) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0 | 0.5 | 0.5 | 0.5 | 0 | 0 |
| Trans. fat (g) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Polyunsat. fat (g) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 1 | 1 | 0.5 | 0 |
| Monounsat. fat (g) | 1.5 | 2 | 1.5 | 1.5 | 2 | 0.5 | 1.5 | 3 | 3 | 3 | 0 | 0.5 |
| Sodium (mg) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Carbohydrate (g) | 21 | 20 | 18 | 18 | 20 | 19 | 17 | 20 | 18 | 19 | 21 | 25 |
| Fiber (g) | 4 | 5 | 3 | 3 | 4 | 5 | 3 | 5 | 5 | 5 | 4 | 3 |
| Protein (g) | 2.5 | 2.5 | 1.5 | 1.5 | 2.5 | 2.0 | 1.5 | 2.5 | 1.5 | 1.5 | 1.0 | 2.0 |
| Vit. C % (RDI 60 mg) | 45 | 45 | 0 | 0 | 30 | 15 | 20 | 15 | 10 | 15 | 25 | 30 |
| Ca % (RDI 1000 mg) | 2 | 3 | 2 | 2 | 4 | 3 | 2 | 3 | 1 | 1 | 1 | 1 |
| Fe % (RDI 18 mg) | 3 | 3 | 0 | 0 | 4 | 3 | 4 | 4 | 4 | 4 | 6 | 4 |
| Source of data: | Labs 2018 | Labs 2018 | Labs 2017 | Labs 2017 | Labs 2018 | Labs 2018 | Labs 2016 | Labs 2016 | Labs 2016 | Labs 2016 | Labs 2016 | USDA database |

In addition to the broader macronutrient analysis described above, some samples were analyzed for a fatty acid profile (Figure 8.4.1a; Appendices VI and VII). This shows the relative amounts of different kinds of fats (fatty acids) that comprise the total fat content of a sample (“Total fat” in Table 8.4.1a above). While we are not aware of fatty acid ratios specifically presenting plant pest risks, substantial changes to chestnut composition due to transgene insertion or expression could potentially affect nutrition value to wildlife, nut decomposition, or other environmental interactions. We found that most American chestnuts, regardless of transgene presence, had similar fatty acid profiles. This supports the conclusion that Darling 58 chestnuts are compositionally similar to related non-transgenic controls, while there is clear variation present between other types of unregulated non-transgenic chestnuts.

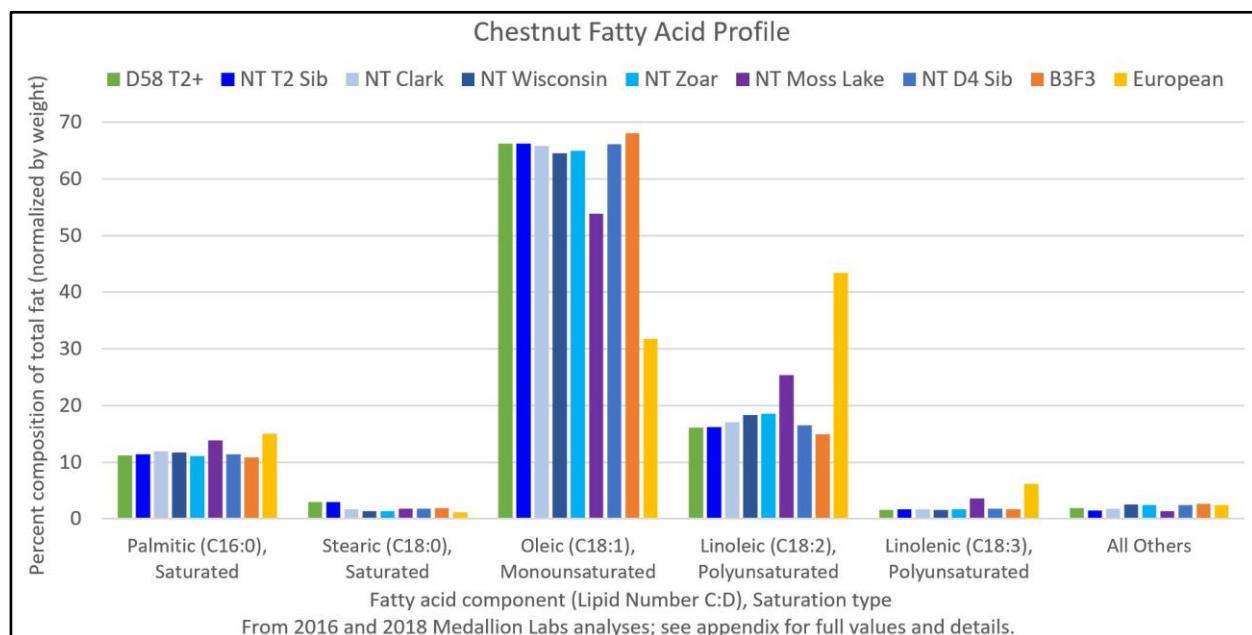


Figure 8.4.1a. Chestnut fatty acid profile, including Darling 58 T2 (green bar at far left of each component), non-transgenic full sibling of T2 (bright blue bar adjacent to green), and several non-transgenic American chestnut controls (NT, other blues and purples). Backcross (B3F3, orange second from right) and European chestnut (yellow, far right) are included for comparison. Height of each bar indicates the percent of total fat in a sample that is comprised of this fatty acid component. Lipid number (C:D) indicates the number of carbon atoms (C) and the number of double bonds (D); fats without any double bonds are considered saturated.

Considering chestnut fatty acid profiles more generally, the European chestnut quantities in our analysis (yellow column) align well with previous profiles reported for this species (Borges *et al.*, 2007; España *et al.*, 2011; Barreira *et al.*, 2012). Two publications reporting fatty acid profiles for American chestnuts (Senter *et al.*, 1994; Anagnostakis, 2009) also generally corroborate our observed ratios; their combined ranges are: 8 – 19% palmitic acid, 54 – 60% oleic acid, 17 – 22% linoleic acid, and ≤ 2% others, all of which are within a few percent of our observed ranges.

8.4.2 Tannin analysis

Tannins are phenolic compounds produced by many plants, often associated with pigmentation and pest defense, and historically used in leather production and other industries. Tannins are

broadly categorized into condensed tannins (also called proanthocyanidins, based on flavonoid monomers) and less numerous hydrolyzable tannins (based on gallic acid) (Schofield *et al.*, 2001; Redondo *et al.*, 2014). Plant tannins can offer environmental benefits to humans in the form of building blocks for bio-based polymers or adhesives (Arbenz and Avérous, 2015; Graichen *et al.*, 2017). Tannin content can be an important factor for the use of chestnuts in animal feed (Liu *et al.*, 2011a; Liu *et al.*, 2011b; Redondo *et al.*, 2014; Buccioni *et al.*, 2015; Bonelli *et al.*, 2018), and is therefore potentially relevant for wild animal consumption as well. Tannins can also affect chestnut susceptibility to insect herbivory (Rieske *et al.*, 2003; Cooper and Rieske, 2008), and microbial communities surrounding leaf litter (Winder *et al.*, 2013), so substantial changes to tannin content could potentially have plant pest risks in the form of environmental interactions with transgenic chestnuts.

We had several types of chestnuts tested for tannin content in two separate analyses. In the first analysis, chestnuts were peeled (shell and majority of pellicle removed) to represent the state in which they are consumed by people. In the second, chestnuts were left unpeeled to represent the state in which they are encountered by animals. (We use the term “peel” to refer to the combined shell and pellicle layers, and to the process of removing those layers in preparation for human consumption.) Samples for each analysis included a subset of the following types: Darling 58 transgenic T2 nuts from two separate mother trees (MA & FA), non-transgenic full-sibling controls from the same mother trees, unrelated wild-type American chestnuts (Moss Lake, Sherburne, and/or Mass), and an open-pollinated Chinese chestnut. All samples were stored at 4°C for approximately two months before testing; transgenic samples were identified by testing a nut sample for oxalate oxidase activity via a histochemical assay (Section 7.4.1).

Each sample contained ~5 – 6 grams of nuts (4 – 6 peeled or 3 – 4 whole nuts) that had been chopped, frozen, ground, and divided into three subsamples. Testing for both analyses was performed by the University of Nebraska’s Natural Product and Food Analysis Facility⁵ in January and February 2019 (Appendix VIII). Extraction consisted of an acid digestion, which generally extracts more proanthocyanidins (condensed tannins) than hydrolyzable tannins (Aires *et al.*, 2016). Mean tannin content (in mg catechin equivalents per gram of fresh tissue) for each sample was calculated from the three analyzed subsamples. Means were compared by Tukey’s HSD; shared letters above the columns in Figures 8.4.2a and 8.4.2b indicate non-significant differences ($p > 0.05$) between sample types.

In both analyses, there are significant differences in tannin content between American chestnuts from different mother trees, but tannin content was not significantly different in transgenic vs. non-transgenic full siblings from a single mother tree (designated FA or MA). Among peeled samples, tannin content of the Chinese chestnut sample was not significantly different from any other sample type, but this is not entirely surprising as other studies have shown that foliar tannin content in chestnuts can vary more by season (Kellogg *et al.*, 2005) or soil treatment (Rieske *et al.*, 2003) than among *Castanea* species or interspecific hybrids.

⁵ Available at: <https://foodsci.unl.edu/natural-product-and-food-analysis-facility>

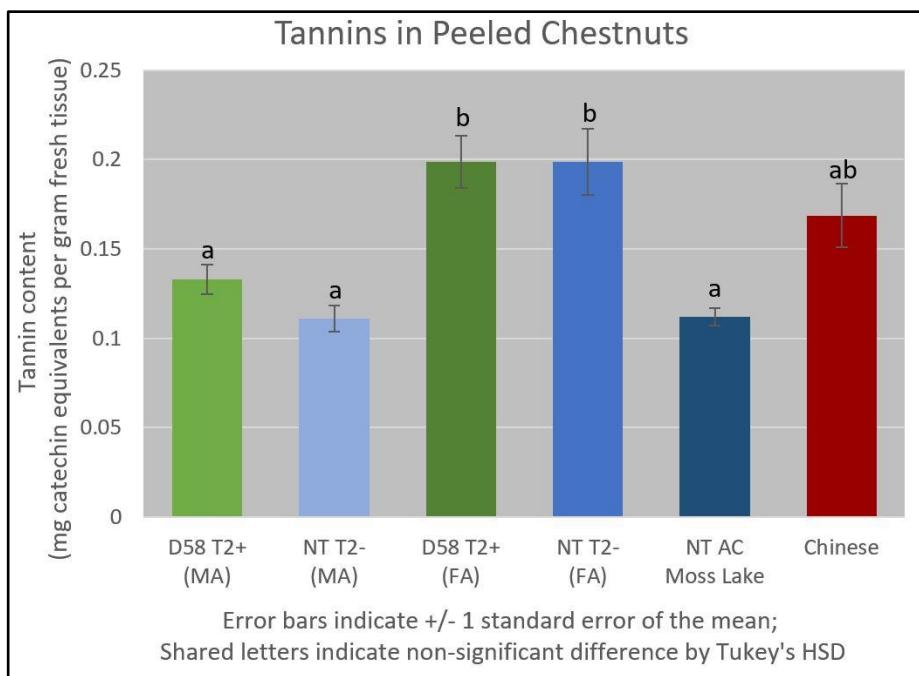


Figure 8.4.2a. Tannin concentration in peeled chestnuts (the state in which they are eaten by humans). “D58 T2+” (green bars) indicates transgenic T2 offspring of Darling 58; “NT T2-” indicates non-transgenic full sibling from the same cross. Two-letter code (MA or FA) indicates mother tree source of T2 cross. “NT AC Moss Lake” is an unrelated American chestnut from a different plot (dark blue bar), Chinese chestnut control (red bar) is from the same plot as T2 crosses.

It is well known that chestnut shell (pericarp) and pellicle (also called skin, endocarp, or testa) tissues contain more tannins than the kernel/nut meat (de Vasconcelos *et al.*, 2010a; Lee *et al.*, 2016), and this was generally reflected by our analysis of whole (unpeeled) chestnuts. Of the samples that were analyzed both with and without peels, tannin levels were consistently higher with peels, but the ratio varied from ~1.3- to ~12-fold higher in samples containing peels. There was significant variation between unpeeled nuts from different sources, but the transgenic and non-transgenic nuts from a single mother tree (FA) were almost identical to each other, and similar to one of the unrelated non-transgenic American chestnut controls. Two of the other non-transgenic American controls showed significantly different tannin concentrations (one higher and one lower than the Darling 58 T2 offspring), again reflecting differences that may be due to genotype background, growing conditions, or environmental variations. The unpeeled Chinese chestnut sample showed relatively low tannin concentrations compared to most American chestnut samples, but this could be attributable to the relative difference in peel:nut ratio in the much larger Chinese nuts, or to well-documented differences in peel tannin content between varieties of chestnut (Hwang *et al.*, 2001; de Vasconcelos *et al.*, 2009).

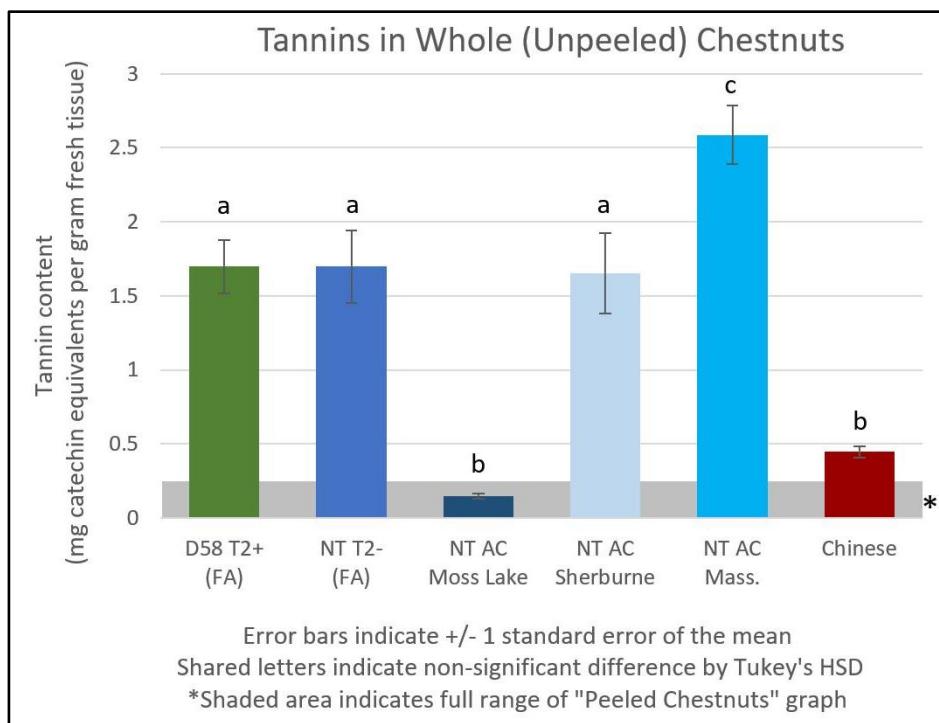


Figure 8.4.2b. Tannin concentration in unpeeled chestnuts (the state in which they are most often encountered by animals). “D58 T2+” (green bar) indicates transgenic T2 offspring of Darling 58; adjacent “NT T2-” is a non-transgenic full sibling from the same cross. (FA) indicates a single mother tree source of T2 offspring, see Figure 8.4.1a. “NT AC...” types are unrelated American chestnuts from different plots (blue bars), Chinese chestnut control (red bar) is from the same plot as T2 crosses.

8.4.3 Potential dietary consumption of OxO from transgenic chestnuts

Although OxO genes are found in many crop plants (Table 4.2a), the major sources in the U.S. diet are likely wheat and corn (maize). Annual wheat consumption in the U.S. has fluctuated over the past century between 110 and 225 pounds per capita. The USDA Economic Research Service estimated U.S. per capita wheat flour use in 2011 at 132.5 pounds⁶. Americans also eat an average of 22.5 lbs of rice, 10.4 lbs of bananas, and 3.5 lbs of strawberries per person every year (Gilbert-Diamond *et al.*, 2011; 2010 ERS data⁷), as well as other OxO-containing crops such as barley, sorghum, beets, apricots, and peanuts. OxO is heat stable, and can be detected in both fresh and cooked foods (Lane, 2000; Kanauchi *et al.*, 2009), so this enzyme is already consumed in its active form without known problems. It is worth noting that oxalate oxidase may be broken down or at least inactivated during digestion, typically requiring modification or encapsulation for use as a medical treatment (Hu, Y. *et al.*, 2015, Cowley and Li, 2017). In addition to human dietary and livestock feeds, OxOs are found in many species of wild plants and fungi that are routinely consumed or encountered by wildlife (Section 4.2).

⁶ Available at: <http://www.ers.usda.gov/topics/crops/wheat/background.aspx>

⁷ Available at: <http://www.ers.usda.gov/data-products/chart-gallery/detail.aspx?chartId=30486>

Chestnuts are flavorful and nutritious, and can be used in a variety of food products. However, they are primarily a seasonal or holiday delicacy rather than a staple of most modern diets. It is useful to compare the above-listed wheat consumption to annual chestnut consumption in countries where chestnuts are widely available: Korea 4.0 lbs, Japan 1.2 lbs, Europe 1.0 lbs, China 0.5 lbs, and the U.S. 0.1 lbs per person (Vossen, 2000). Therefore, even if chestnut consumption in the U.S. grew to match the highest rate in the world (Korea, at 4.0 lbs) and people consumed exclusively transgenic OxO chestnuts, OxO consumption would likely still come predominantly from wheat and other sources.

OxO consumption in a single serving of transgenic chestnuts (i.e. acute exposure) would likely be higher than that from a single serving of wheat or other foods, given the relatively higher expression of OxO in Darling 58 chestnuts (Section 7.4.2). A 50-g serving of fresh Darling 58 American chestnuts might have approximately 8 mg of OxO (assuming ~0.3 mg OxO per gram of dry nut tissue, see Figure 7.4.2a, and assuming ~50% moisture content of fresh nuts, for a final quantity of ~0.16 mg OxO per gram of fresh nut tissue). Since dosage rates or nutritional recommendations have not been established for OxO, we can look to medical applications (for treatment of hyperoxaluria) to get an idea of acceptable quantities in mammalian systems. Dosage rates of crystalized oxalate oxidase as a medical treatment have been suggested at up to 100 mg OxO per kg of body mass (Shenoy *et al.*, 2008), or 7.5 g of oxalate oxidase for a 75 kg adult human, with no apparent concerns for toxicity. This is nearly 1000 times more OxO than someone would consume in a serving of transgenic chestnuts. A separate experiment on rats involved injection of capsules containing 24 enzyme units of OxO per ~100 g rat, and reported no harmful effects, but rather beneficial reductions in harmful oxalate (Raghavan and Tarachand, 1986). Based on a commercial purified OxO product (Sigma-Aldrich item #O412724), activity can be approximately 0.5 – 1.5 units/mg. Thus 24 units of OxO is equivalent to ~16 – 48 mg of purified OxO in a 100 g rat. A proportional dose would be ~12 – 36 g of OxO for a 75 kg adult human; approximately 2000 to 4500 times more than that found in a serving of transgenic chestnuts. See Section 8.4.5 for additional examples of medical OxO use. These examples suggest that acute exposure to OxO should not be harmful to people or other mammals, even at quantities more than three orders of magnitude higher than would likely be consumed in transgenic chestnuts.

8.4.4 Lack of allergenicity

The Darling 58 American chestnut tree does not represent a source of new, potentially allergenic or anti-nutrient proteins; the oxalate oxidase gene and protein are commonly found in a variety of non-allergenic foods (Table 4.2a). The OxO enzyme (and its encoding gene) is eaten by over a billion people daily in wheat and other grains, and has not been identified as an allergen in any known reports. Additionally, the fact that the OxO enzyme is present in corn, rice, sorghum, and many other foods that are *not* considered allergens (FDA, 2006; Hefle *et al.*, 1996; Table 4.2a) provides logical support for a lack of allergenicity in OxO.

Chestnut *pollen* can be allergenic, especially to those with allergies to latex (Wagner and Breiteneder, 2002) or other tree pollen (Hirschwehr *et al.*, 1993). OxO has not yet been quantified in Darling 58 pollen, but based on studies of the CaMV-35S promoter in other transgenic plants, transgene expression in pollen will likely be very low or negligible (Section 9.1.4). But even if OxO is meaningfully expressed in transgenic chestnut pollen, there is no reason to expect the presence

of OxO would modify its allergenicity. One study observed presence of increased immunoglobulin (IgG) titers when OxO was injected directly into the bloodstream of mice (Zhao *et al.*, 2018). This would be expected of *any* foreign protein introduced intravenously, and does not represent likely exposure routes of transgenic plant products.

Oxalate oxidase belongs to the extremely diverse superfamily of proteins known as cupins (Dunwell *et al.*, 2004), which also includes globular storage proteins known as globulins. A number of globulins have been identified as major plant allergens in peanut, walnut, and soybean (Jensen-Jarolim *et al.*, 2002). However, the more specific family of cupins to which OxO belongs, germin-like proteins, has been reported as non-allergenic, separate from cupin families that are known to include allergens (Radauer and Breiteneder, 2007).

Potential allergenicity of the OxO transgene was investigated by querying the protein's amino acid sequences via multiple databases (see below) to verify that the sites responsible for allergenicity in other cupins were not present on the OxO enzyme. Some unrelated cupins with similar enzymatic activity to OxO have been shown to induce allergic symptoms in patients with wheat allergies (Jensen-Jarolim *et al.*, 2002). However, the OxO enzyme we used in chestnut does not match these cupins in amino acid alignments (see below) and is a completely separate enzyme belonging to a different sub-family (Radauer and Breiteneder, 2007). Other main allergens in wheat, an α -amylase inhibitor (Breiteneder, 1998), and omega-5 gliadin (Ito *et al.*, 2008) are also completely unrelated to OxO and their amino acid sequences do not have any matches with OxO. Gluten is another concern for some people; see Section 11.5 for a discussion of the lack of association with gluten in transgenic chestnuts.

Database queries on the specific wheat OxO used in Darling 58 were performed using the OxO amino acid (AA) sequence (Table 7.1.1a) in databases of known allergens using three separate tests of varying stringency. A recommended test for determining allergenicity of novel food products involves screening for identity matches in a “sliding window” of 80 amino acids. The Food and Agricultural Organization of the United Nations (FAO) and World Health Organization (WHO) have set a threshold for cross-reactivity with known allergens of 35% AA identity matches within any 80-AA sequence, or 6 contiguous matching amino acids (FAO, 2001). Researchers have subsequently suggested that a match of 6 contiguous amino acids may not actually be relevant, since there can be many false positives to non-allergenic foods with this type of comparison; matches of 8 contiguous amino acids are still stringent but reduce false positives (Silvanovich *et al.*, 2006). More recent recommendations have suggested a full-length alignment by FASTA (Lipman and Pearson, 1985) may reveal more relevant similarities with fewer false positives than the 80-AA sliding window approach, and that the 35% identity threshold might actually be too low (Ladics *et al.*, 2007). Both of these recommendations would indicate the standard tests might be unnecessarily stringent, matching to sequences that aren't actually allergenic, but results of all three test types (as performed in January 2019) are presented here.

We first used the University of Nebraska's AllergenOnline interface to the “FARRP” allergen protein database (version 18B released on March 23, 2018⁸). A query with the wheat OxO amino acid sequence revealed no significant matches to any known allergens, including wheat allergens,

⁸ Available at: www.allergenonline.org/about.shtml

by any of the three test types. Results from the 80-AA sliding window search (no matches) are shown in Figure 8.4.4a. Full FASTA searches showed the closest match to have less than 24% shared AA identity an E-value > 0.02. Smaller E-values indicate more significant matches: values less than 0.01 are generally considered potentially homologous, and less than 10^{-6} indicate high significance of a match; Schein *et al.*, 2007. No 8-AA exact matches were found.

| 80mer Sliding Window Search Results | |
|--|---|
| Database | AllergenOnline Database v18B (23 March 2018) |
| Input Query | >query MGYSKTLVAGLFAMLLAPAVLATDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFL FSSKLAKAGNTSTPNGSATVELDVAEWPGNTLGVSMNRVDFAFGGTNPPIHPRATEIG IVMKGEELLVGILGSLDSGNKLYSRVVRAGETFLIPRGLMHFQFNVGKTEASMVVFNSQN PGIVFVPLTLFGSNPPPIPTPVLTKALRVEARVVELLSKFAAGF |
| Length | 224 |
| Number of 80 mers | 145 |
| Number of Sequences with hits | 0 |
| No Matches of Greater than 35% Identity Found | |
| AllergenOnline Database v18B (23 March 2018) | |

Figure 8.4.4a. AllergenOnline database results confirming no amino acid matches above 35% identity with known allergens.

For more detailed results on the commonly recommended 80-AA sliding window test, we used a different database interface called the Structural Database of Allergenic Proteins (SDAP⁹, last updated February 25, 2013; Ivanciu *et al.*, 2002; Ivanciu *et al.*, 2003). According to the 80-AA sliding window comparison performed with SDAP, the closest identity match of a known allergen to the wheat OxO is 22.5% (18 amino acids in an 80-AA window), with a maximum consecutive match of two amino acids in the same window. This is well below the conservative concern threshold of 35% within an 80-AA window. Full FASTA and exact 8-AA comparison results using the SDAP database were similar to those from AllergenOnline.

Based on the presence of OxO in many foods not considered allergenic, the lack of reported allergenicity of germin-like proteins as a family, and a lack of significant cross-reactivity matches from allergenicity database searches, we conclude that the wheat oxalate oxidase enzyme is not a known or suspected allergen.

8.4.5 Lack of toxicity

Oxalate oxidase and similar enzymes are consumed and handled daily in cereal grains and many other foods without any reports of toxicity, and we have found no evidence that it should be considered a toxin. Many OxO-containing food crops as described in Sections 4.2 and 8.4.3 are cultivated, harvested, processed, packed, and consumed worldwide by humans (and eaten by animals), with no known reports of harm (allergenicity or toxicity) due to contact with the OxO enzyme. Apart from ingestion in food, skin contact with OxO-containing grain and grain products regularly occurs during harvest, processing, and baking. Products with ingredients from OxO-containing plants such as oats and apricot pits are found in skin care products (Kurtz and Wallo, 2007; Allemand and Baumann, 2009; Dona *et al.*, 2015), again with no reported toxic or allergenic

⁹ Available at: fermi.utmb.edu/SDAP/index.html

properties. Oxalate oxidase has been proposed and tested for use in food packaging (Winestrand *et al.*, 2013), medical treatments to reduce harmful oxalate levels (Raghavan and Tarachand, 1986; Dahiya and Pundir, 2013; Hu, Y. *et al.*, 2015; Cowley and Li, 2017; Zhao *et al.*, 2018), and coatings for medical devices prone to oxalate buildup (Malpass *et al.*, 2002; Mellman, 2007; Shepard, 2008), all with no apparent concerns regarding toxicity or dietary allergenicity. We are aware of only one study directly testing potential toxicity (“biocompatibility”) of oxalate oxidase with mammalian cells (NIH/3T3 mouse cells), which concluded “cell viability was maintained at approximately 100%” regardless of OxO concentration (Zhao *et al.*, 2018). More anecdotally, dozens of researchers at ESF and elsewhere over the past ~25 years have planted, handled, measured, harvested, and generally interacted with OxO-expressing transgenic plants, with no observable negative effects.

As described previously, OxO detoxifies oxalate, which *is* a known toxin to humans and other organisms (Christison and Coindet, 1823; Brown and Gettler, 1922; Hodgkinson, 1977; Svedružić *et al.*, 2005), leading to conditions such as hyperoxaluria and kidney stones (Massey *et al.*, 1993; Noonan and Savage, 1999; Ogawa *et al.*, 2000; Rahman *et al.*, 2013).

In order to more specifically screen the oxalate oxidase expressed in Darling 58 chestnuts against known toxins, we queried the “Toxin and Toxin Target Database” (soon to be referred to as the “Toxic Exposome Database”) found at www.t3db.ca (Lim *et al.*, 2010; Wishart *et al.*, 2015). Multiple search functions were employed, most recently in January 2019, including a Sequence Search (for both amino acid and DNA sequences of wheat OxO, Table 7.1.1a), and a Text Query for “oxalate oxidase”. In each case no matches were returned (see example in Figure 8.4.5a). In contrast, a text query for “oxalic acid” returns a match confirming human toxicity of the chestnut blight toxin as found in plants and household or industrial products. Based on results from this toxin database search and because the OxO enzyme is ubiquitous in food, feed, and many other organisms, we conclude that the wheat oxalate oxidase enzyme is not a known or suspected toxin.

The screenshot shows the T3DB website interface. At the top, there is a green navigation bar with the T3DB logo, followed by links for Browse, Search, Downloads, About, and Contact Us. A search bar contains the query "oxalate oxidase" and a green "Search" button. Below the navigation, the main content area is titled "Search Results for compound". A pink message box states, "Searching compound for \"oxalate oxidase\" returned no results." Below this, a grey box titled "Did you mean?" lists three suggestions: "oxalate oxide", "oxinate oxide", and "oxolane oxide". At the bottom of the content area, a small note says "No compounds found".

Figure 8.4.5a. Screen shot from Toxin & Toxin Targets Database website, confirming no matches for “oxalate oxidase” in known toxin databases. Similar results from DNA and amino acid searches are not shown.

9.0 Environmental interactions of Darling 58 American chestnut

Restoring American chestnuts to their native environments could benefit people and ecosystems, but evaluation of potential non-target environmental effects is an important consideration before restoration material is released. Due to the ubiquity of oxalate oxidase in crops, wild plants, mosses, bacteria, and fungi (Section 4.2), it is very unlikely to have an adverse environmental impact when expressed in chestnut, but studies like these are prudent before any new product is widely planted in nature. This section describes assessments of environmental interactions with Darling 58 American chestnuts compared to non-transgenic controls. Tests include laboratory, greenhouse, and field evaluations. Additional tests on other OxO-expressing transgenic American chestnut events are described in Section 10, which provide additional data and supporting evidence for some of the conclusions drawn directly from studies on Darling 58. This section does include some data from other transgenic events only when they were part of the same experiment as Darling 58, but if data are specifically intended for bridging to Darling 58, it is noted and also described in Section 10.

Summaries of each study on Darling 58 are presented here; full reports (in the form of publications, draft publications, reports, posters, or theses) are found in the supplied reference list. The data from these interaction experiments overwhelmingly support a conclusion that Darling 58 American chestnuts are not meaningfully different in terms of environmental interactions than wild-type or traditionally bred controls, with the exception of the blight tolerance trait. This provides further evidence that Darling 58 American chestnuts do not pose novel pest risks to other organisms or ecosystems.

9.1 Non-target interactions

9.1.1 Mycorrhizal colonization of Darling 58 roots

In nature, fine roots of most plants are colonized by fungi in a mutualistic symbiosis (Smith and Read, 2008). Plants provide their fungal partners with sugar, and fungi provide their plants phosphorus, nitrogen, and other mineral nutrients. It is generally accepted that the vast majority of land plants are normally mycorrhizal, and plants such as American chestnut require these fungi for normal growth. Given the importance of mycorrhizal fungi to American chestnut, it is particularly important to demonstrate that transgenic trees which can tolerate fungal infection above ground will still form partnerships with mutualistic fungi below ground. Following are excerpts from Newhouse *et al.* (2018).

Disruptions to mycorrhizal relationships due to breeding or genetic engineering could potentially indicate novel environmental risks. The lack of direct antifungal activity by oxalate oxidase suggests risks to mycorrhizal fungi are unlikely, but careful evaluation of restoration material is prudent. In contrast to pathogenic fungi that employ oxalic acid for a virulence factor (Rigling and Prospero, 2018), mycorrhizal fungi depend on the mutual flow of materials between themselves and their plant hosts, and thus have no direct need for the action of oxalic acid, so its degradation should have no effect on mycorrhizal colonization. Beyond the transgene product itself, the byproducts from oxalate oxidase degradation of oxalic acid are hydrogen peroxide and carbon dioxide (Lane *et al.*, 1993). Hydrogen peroxide has fungicidal properties at sufficiently high concentrations (Baldry, 1983; El-Gazzar and Marth, 1988), but as explained in Sections 4.3 and

6.3.2, the molar concentration of hydrogen peroxide produced by oxalate oxidase will logically never exceed the concentration of oxalic acid produced by the blight fungus. Importantly, chestnut blight does not typically infect tree roots (Hepting, 1974; Weidlich, 1978), so it is unlikely that substantial oxalic acid degradation (and thus hydrogen peroxide formation) would take place in the rhizosphere.

In order to evaluate root tip mycorrhizal colonization of Darling 58 compared to the isogenic line Ellis, a greenhouse bioassay was performed in the spring of 2017. A different event called Darling 54 (Section 10.1) was also included in this experiment and results are described concurrently, but Darling 54 is not necessarily intended for bridging data with Darling 58. Soil samples containing wild fungal propagules for mycorrhizal inoculum (Dulmer *et al.*, 2014) were collected from an experimental plot near Syracuse NY, in a mixed hardwood forest with sugar maple, American beech and Eastern hemlock. Twenty-three samples were taken using a cylindrical soil core 4 cm diameter driven to a depth of 15 cm. The location of each sample within the plot was randomly determined. Soil samples were dried, then sifted through a 0.5 cm mesh (USA Standard Soil Sieve). The soil inoculant was mixed at a ratio of 1:1:2 dried soil: sand: sphagnum peat moss. The resulting inoculant mix was split evenly among 45 pots (D40 Deepots, Stuewe & Sons), which had been previously sterilized overnight in a 7% bleach solution. Tissue culture-generated *C. dentata* approximately six months old were transplanted into pots containing the inoculant. Three types of *C. dentata* were used: 15 individuals each of Ellis, Darling 54 and Darling 58. These were grown in a greenhouse at 21 – 26 °C, with a 16-h light/8-h dark cycle, and watered as needed. In order to encourage associations with mycorrhizal fungi, the plants did not receive fertilizer or pH amendments during the experiment.

Mycorrhizal colonization rate was assessed after 5 months of growth by collecting a continuous root length of at least 15 cm from each surviving plant. All root tips along the collected sample were observed, and the percentage of root tips with evidence of a fungal mantle, and those without a mantle, were visually estimated using a dissecting microscope and assigned to categorical percentage ranks (e.g. 90 – 95% or > 95%). A root tip was considered ectomycorrhizal if it was actively colonized (Figure 9.1.1a) or senescent with indications that it had been previously colonized. The frequencies of each category in each treatment were calculated. A Fisher's exact test of independence with a significance of 0.05 was used to test the null hypothesis that there was no significant difference in root tip colonization between any two treatments.



Figure 9.1.1a. Ectomycorrhizal (EM) root tips harvested from American chestnut seedlings. Plant roots are seen on the left side of both photos as a brownish main (secondary) root. EM roots branch off of these secondary roots and are wrapped in a fungal sheath or mantle. The fungal mantle gives the EM root tips their distinctive colors, with a white morphological type (morphotype) at left and a golden morphotype at right. Note the hyphal cords in the left photo: these are aggregations of hyphae that facilitate transport of sugars from the plant to the fungus in the soil and soil nutrients from the fungus to the plant roots. From Newhouse et al. (2018); photos courtesy T. Horton.

Surviving trees included 15 Ellis, 10 Darling 54, and 12 Darling 58. Mycorrhizal colonization was consistently high among all types, with *all* observed roots on surviving Ellis and Darling 58 plants showing greater than 95% ectomycorrhizal colonization. The observed root from one Darling 54 tree showed 90 – 95% colonization, with the remainder showing > 95% colonization. According to Fisher's exact tests, there were no significant differences in colonization between Ellis and the transgenic lines Darling 54 ($p = 0.400$) or Darling 58 ($p = 1.00$).

This supports previous investigations on the mycorrhizal condition of other transgenic American chestnuts based on laboratory (D'Amico *et al.*, 2015) and field (Tourtellot, 2013) bioassays (Sections 10.6.1 and 10.6.2, respectively). Even with the higher OxO transgene expression in Darling 54 and Darling 58 compared to the Darling 4 tested previously, there were no significant differences in colonization by ectomycorrhizal fungi in roots compared to non-transgenic controls. More generally, these results suggest that presence or expression of OxO in Darling 58 does not pose novel pest risks to native soil fungi that are ecologically important for American chestnuts and other trees.

9.1.2 Responses of plants found in chestnut habitats: germination of seeds in Darling 58 leaf litter

In order to observe effects of chestnuts on surrounding plants in a controlled environment, a greenhouse bioassay was performed to evaluate interactions between leaf litter from transgenic American chestnut trees and seeds from other species that are native or naturalized in American chestnut habitats (Newhouse *et al.*, 2018). This was tested by germinating several types of seeds in a tray of potting mix containing dried, crushed chestnut leaves. Seed types included a grass (*Elymus virginicus*), a forb (*Cichorium intybus*), a shrub (*Gaultheria procumbens*), a coniferous tree (*Pinus strobus*), and a deciduous tree (*Acer rubrum*), all of which are native to (or naturalized in) American chestnut habitats. Three other seed types were started, but did not germinate appreciably in any tray, so were not included in subsequent analyses. All seeds were purchased from a regional supplier (Sheffield's Seed Company, Locke NY), and had been stratified by the supplier.

Leaf types included two non-transgenic American chestnuts (McCabe Hollow and Ellis), two transgenic American chestnuts (Darling 54 and Darling 58), traditionally bred hybrid and backcrossed chestnuts (F1 and B3F3), Chinese chestnut (Qing), and a no-leaf control. As with the mycorrhizae study above (Section 9.1.1), Darling 54 was included in this experiment but is not specifically intended to serve as bridging data. All leaves were collected from a permitted field plot near Syracuse NY in the fall of 2016, dried at room temperature for approximately 6 months, and chopped to approximately 1 cm squares. Each tray (~25 cm x 51 cm x 6 cm, with drainage holes) contained 20 g of a single chestnut leaf type mixed into 5 liters of pre-moistened peat-based commercial potting mix. Twenty seeds of each type were planted in a row in every tray, and three replicates of each tray were included. Trays were kept in a greenhouse, arranged in replicated blocks along a long table, and watered weekly or more frequently if needed.

Germination observations, conducted twice weekly, consisted of counting the total number of seeds that had germinated of each type in each tray (Figure 9.1.2a). At the conclusion of observations for a given species, all germinated seedlings were removed from the tray, tapped and brushed gently to remove loose potting mix, dried at 60 °C in a paper bag for 48 hours. Total seedling dry biomass was recorded for each species in each tray. This was conducted at ~4 weeks for the relatively fast-growing *Cichorium*, and 8 – 10 weeks for remaining species. Mean counts and masses from each tray were analyzed with one-way ANOVA (GLM Procedure, SAS v9.2, SAS Institute, Cary, NC, United States) and compared using Tukey's Studentized Range (HSD) test ($\alpha = 0.05$).



Figure 9.1.2a. Germination study tray with leaf litter pieces and germinating seedlings. Inset (right) shows plant roots growing through a chestnut leaf piece, confirming interactions between seedling roots and leaf litter. From Newhouse et al. (2018).

There were no significant differences in seed germination in the presence of transgenic vs. non-transgenic American chestnut leaf litter, in terms of numbers of seeds germinated or total dry biomass of germinated seedlings. Mean counts and masses of germinated seedlings in all leaf types are shown in Figure 9.1.2b. Tukey's HSD test indicated only a few pairwise comparisons with statistically significant differences (presented here as mean \pm 1 SE). Count of *Pinus* seedlings was significantly different between McCabe Hollow (7.0 ± 1.2 germinants) and the no-leaf control (17.0 ± 2.5 germinants), and the mean biomass of *Cichorium* seedlings was significantly different between Darling 58 (1.52 ± 0.19 g) and B3F3 (0.83 ± 0.14 g). These few statistically significant differences in seedling germination between leaf types (*Pinus* counts, *Cichorium* mass) did not

represent trends between transgenic and non-transgenic American chestnuts. There were no significant differences between either of the transgenic leaf types and the non-transgenic Ellis control, which is genetically identical to the Darling lines in this experiment other than transgene presence. Additionally, allelopathy by chestnut leaves in general was not broadly apparent, as no-leaf control trays showed overall similar germination for most seed species. Collectively, these studies reinforce previous and concurrent findings that transgenic American chestnuts are not substantially different than non-transgenic American chestnuts (apart from their enhanced blight tolerance), and do not pose a novel pest risk to several types of representative native plants.

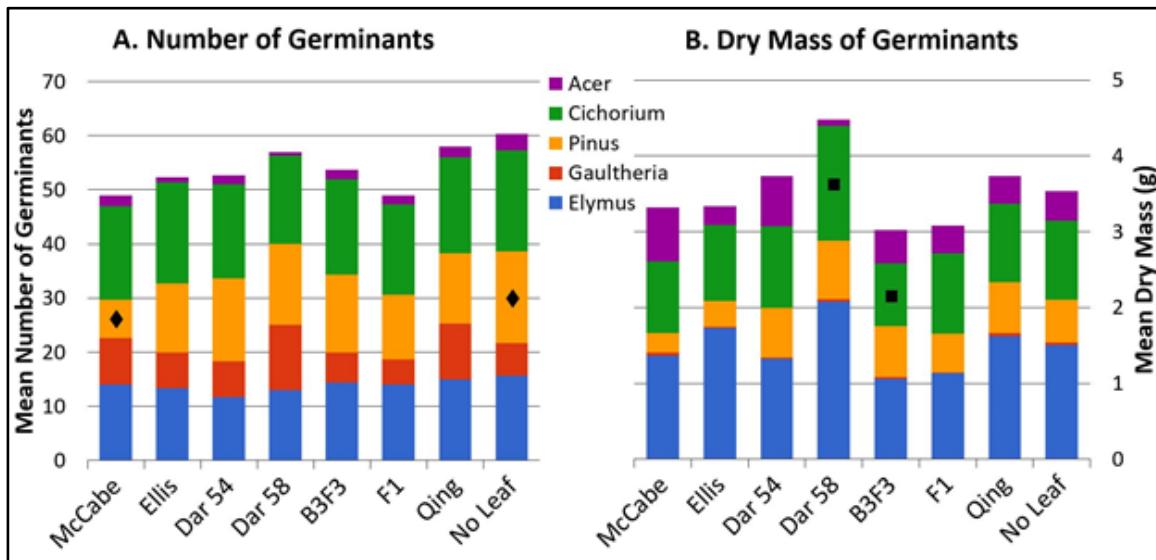


Figure 9.1.2b. Total mean counts (A., n = 20 seeds/type) and dry masses (B.) of germinated seedlings for all leaf and seed types. Statistically significant differences for pairwise comparisons within seed species are noted with ♦ (Pinus counts, McCabe vs. No Leaf) and ■ (Cichorium mass, Darling 58 vs. B3F3). Dar 54 = Darling 54, Dar 58 = Darling 58, see text for explanations of other leaf types. From Newhouse et al. (2018).

9.1.3 Insect herbivory on chestnut leaves

Although there are a few reports of insect resistance by some OxO-expressing plants as described in Section 4.5 (Ramputh *et al.*, 2002; Mao *et al.*, 2007), interactions between chestnuts and herbivorous insects tested to date are not significantly affected by OxO (see below, and Sections 10.6.6 and 10.6.7). Indirect effects such as H₂O₂-driven insect interactions and nutritional benefits due to oxalate breakdown (Kumar *et al.*, 2016) would only apply in cases where oxalic acid is prevalent in the tissue product: outside of blight infections; vegetative or edible portions of American chestnuts do not have measurable levels of oxalic acid, so OxO would therefore not produce hydrogen peroxide. This is demonstrated by a lack of color change in “-” tubes of oxalic acid histochemical assays on transgenic chestnut tissues (Figures 7.4.1b and 9.4b). If oxalic acid were present in these samples, OxO-expressing tissues in the “-” tubes would stain blue-black, indicating the generation of hydrogen peroxide from oxalic acid breakdown (Section 6.3.2).

A series of tests were done to evaluate gypsy moth feeding on various transgenic lines including Darling 58 (Brown, 2016; Brown, 2017; Brown *et al.* 2019). Since forest management practices for invasive insects can include application of biocontrols, these tests primarily investigated potential

unintended tritrophic impacts of transgenic American chestnut on plant/herbivore/biocontrol interactions. During the study, gypsy moth (*Lymantria dispar*) caterpillars were fed chestnut leaf discs that were treated with one of two biocontrols: either the species-specific *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV) or the generalist pathogen *Bacillus thuringiensis* subsp. *Kurstaki* (Btk). Resulting caterpillar mortality was quantified and compared to control leaf discs treated with water. These insect pathogens are important regulators of phytophagous lepidopterans, and there could be plant pest risk concerns if insect feeding on treated transgenic leaves was substantially different than that on non-transgenic controls, or if transgene presence inhibited the effectiveness of a biocontrol treatment.

Chestnut leaf discs (including Darling 58, Ellis, Chinese, Ashdale, and backcross) were collected from greenhouse-grown chestnuts, and treated with an LD₅₀ dose of biocontrol formulations approximately similar to those that are used to control gypsy moth infestations in forests (or a water control treatment). The Ashdale line used in this study has mostly American chestnut characteristics, and so was considered a wild-type American chestnut during the study. However, according to a subsequent preliminary genetic analysis involving single nucleotide polymorphisms (unpublished), Ashdale appears to be approximately 75% American chestnut and 25% European chestnut, and therefore could be considered an American x European hybrid. Additionally, Brown (2016) refers to BC3F3 backcross chestnuts as hybrids, which is not consistent with TACF literature (e.g. Steiner *et al.*, 2017). In this summary we are following the usage (e.g. "American" for Ashdale) in the Brown (2016) publication, but specifying "backcross" instead of "hybrid" where appropriate. Gypsy moth larvae were reared on a standard artificial caterpillar diet until they molted to second instar (i.e. equal development stage), at which point they were kept in containers without food for 24 hours before being exposed to treated chestnut leaves for 24 hours. Cumulative caterpillar mortality was observed 14 days after treatment, and percent leaf disc consumption was calculated.

All analyses were performed in R (R Core Team, 2014). Estimates of the LD₅₀ and 95% confidence intervals were calculated using a generalized linear model (GLM) using the MASS package (Venables & Ripley, 2002). Because interest lay in an interaction between leaf material and entomopathogens, only caterpillars consuming ≥25% of the leaf disc were included for analysis of the Btk LD₅₀.

Some significant differences in leaf herbivory were observed between various leaf types, however most of these differences were between the Chinese or backcross chestnuts and the Ashdale or Ellis American chestnut types (Figure 9.1.3a). There were no significant differences between Darling 58 and Ellis American chestnut controls in evaluations of biocontrol efficacy (i.e. caterpillar mortality on different leaf types). The Btk biocontrol treatment showed a significant mortality difference between Darling 54 and Ellis, but Darling 54 was not significantly different than any of the other leaf types in this assay. There were also significant differences in consumption of Btk-treated leaves between Chinese and American leaf types, but no significant differences in consumption between Darling 58 and non-transgenic American controls (Figure 9.1.3b). Overall, these tests confirmed that insect herbivory on OxO-expressing transgenic American chestnuts is not significantly different than that on non-transgenic controls, and furthermore, that Darling 58 transgenic leaves do not differentially affect biopesticides or parasitoids used to control insect pests.

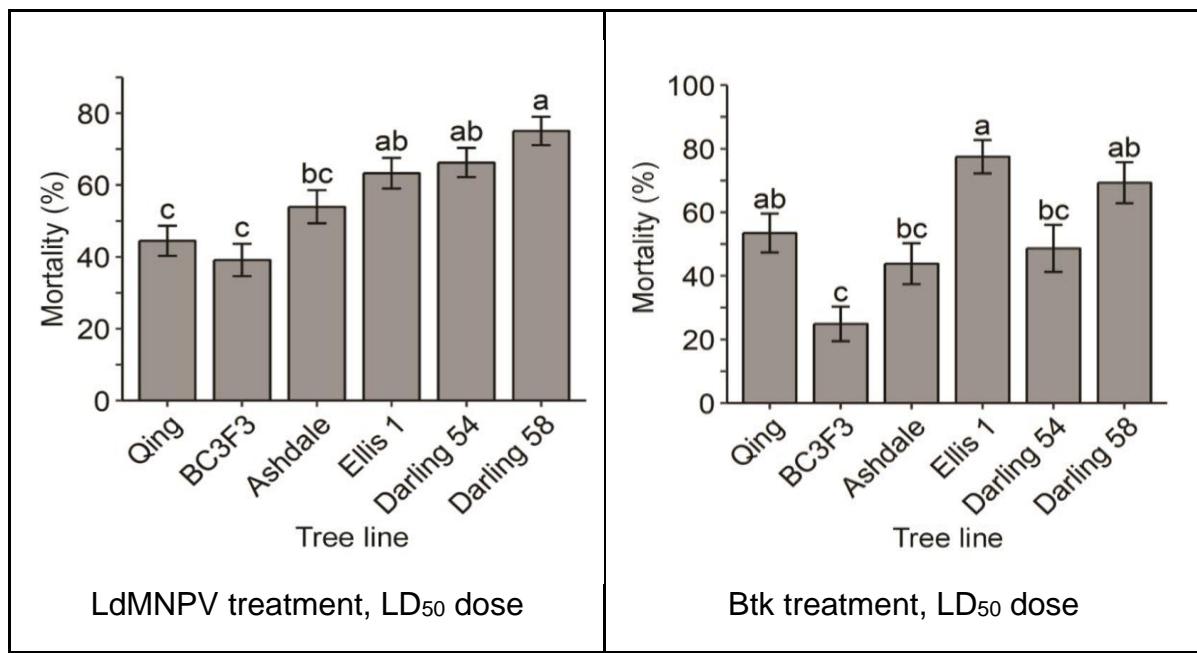


Figure 9.1.3a. Gypsy moth caterpillar mortality after consuming chestnut leaves treated with LdMNPV (left) and Btk (right). LD₅₀ dose was calculated on Ashdale leaves; 50% mortality would be expected. Columns sharing the same letters are not significantly different ($p > 0.05$; Tukey's HSD). Error bars indicate +/- one standard error of the mean. From Brown et al. (2019).

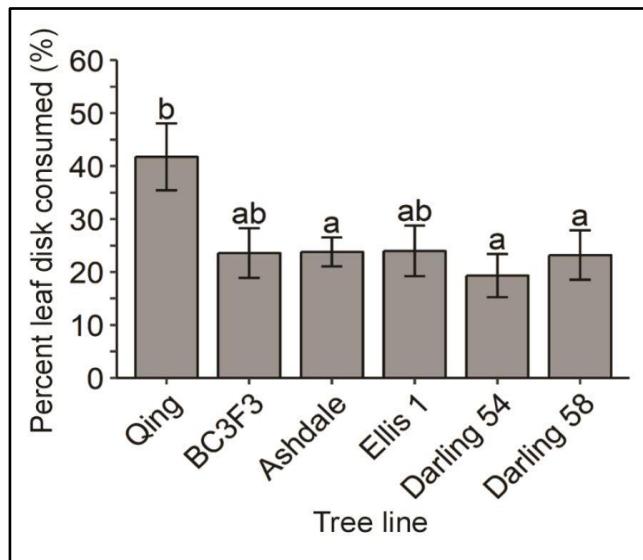


Figure 9.1.3b. Mean consumption of Btk-treated chestnut leaves by gypsy moth caterpillars. Columns sharing the same letters are not significantly different ($p > 0.05$; Tukey's HSD). Error bars indicate +/- one standard error of the mean. From Brown et al. (2019).

Insect herbivory of native trees and potential interactions with biocontrol treatments both have implications for restoration. As an example, Rieske *et al.* (2003) concluded that gypsy moth performance (growth rate and leaf consumption per caterpillar) was better on hybrid chestnuts than on American chestnuts, possibly due to a trade-off between susceptibility from insect herbivory and tolerance of blight in hybrids. A follow-up study (Kellogg *et al.*, 2005) tested

multiple hybrid types, and found some herbivory differences between hybrids of different backgrounds, but no clear correlation between blight resistance and herbivore susceptibility. These studies correlate well to Brown's (2019) reported herbivory differences among chestnuts of different species origins, and collectively they suggest that the presence of OxO in Darling 58 chestnuts has a smaller impact on herbivory or biocontrol interactions than traditional breeding.

9.1.4 Bumble bees and pollen with OxO

The following study, described in greater detail in a pending publication (Newhouse *et al.*, in prep.), evaluated interactions between native bees and chestnut pollen containing the OxO enzyme. Bumble bees forage on chestnut pollen and likely contribute to chestnut pollination (Manino *et al.*, 1991; de Oliveira *et al.*, 2001; Hasegawa *et al.*, 2015; Tumminello, 2016; Zirkle, 2017), so they could be exposed to OxO in pollen during restoration efforts. Real-world exposure of pollinators to OxO depends on transgene expression in pollen, which was not feasible to measure in currently available quantities of transgenic pollen. Studies on other transgenic plants suggest that transgene expression controlled by the 35S promoter is negligible in pollen, or expressed at a lower rate than vegetative tissues (see below in this section). Due to limitations on pollen production by transgenic trees, purified barley OxO enzyme (Roche Diagnostics, Mannheim, Germany) was added to non-transgenic chestnut pollen for this experiment. Barley OxO amino acid sequences share 98% identity with those of the wheat OxO transgene in chestnut (Lane *et al.*, 1993), and both sources show similar enzymatic activity in laboratory assays (Lane, 2000; Matthews, personal communication). Activity of the barley OxO enzyme as added to pollen was confirmed both before and after this experiment by histochemical assay (Section 7.4.1).

A total of 27 small colonies (known as "microcolonies," see Figure 9.1.4a) each containing five worker bumble bees (*Bombus impatiens*) were established for this experiment. Each microcolony was constructed of two 473 mL (16 oz.) plastic take-out containers with screen mesh bottoms, containing a 10 mL cup of sucrose water on one side, 200 mg of treated pollen on the other side, and a tube so bees could freely pass between both sides. Microcolonies were provided with American chestnut pollen containing one of three concentrations of oxalate oxidase: a no OxO control, a standard (conservative field-realistic) OxO concentration similar to that found in other transgenic chestnut tissues (Section 7.4.2), and an artificially high concentration (ten times higher than standard). Bees in each microcolony originated from one of three commercial source hives (BioBest, Leamington ON). Similar microcolony studies are employed to study pollinator responses to pesticides or natural plant compounds. This setup allows social interactions among bees and observable reproductive effort, in manageable replicated experimental units (Babendreier *et al.*, 2008; Manson and Thomson, 2009; Gradish *et al.*, 2013).

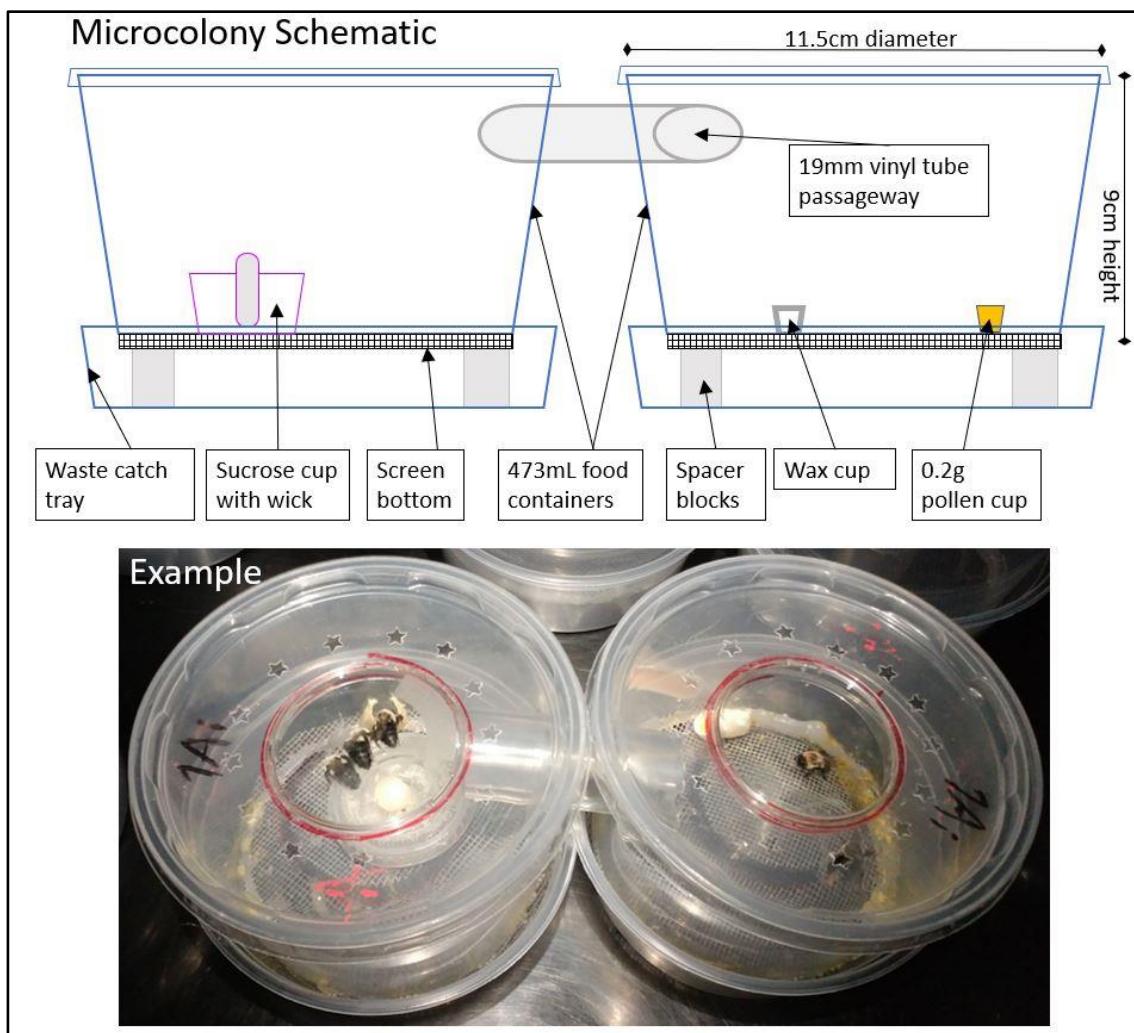


Figure 9.1.4a. Schematic (above) and example (below) of a bumble bee microcolony setup. From Newhouse et al. (in prep.)

It is worth noting that even the standard O_xO concentration in this pollen experiment is likely an overestimate of enzyme levels likely to be found in transgenic chestnut pollen. The nominally constitutive CaMV-35S promoter has actually been found to express transgenes at very low or negligible levels in pollen from many transgenic plants (Twell *et al.*, 1989; Guerrero *et al.*, 1990; van der Leede-Plegt *et al.*, 1992; Mascarenhas and Hamilton, 1992; Wilkinson *et al.*, 1997; Sunilkumar *et al.*, 2002; Hraška *et al.*, 2008; Yan *et al.*, 2015). Only one published source was found (de Mesa *et al.*, 2004) indicating that 35S expression in pollen can be as high as other tissues in strawberry plants, and even this was only in a few of the tested events. No sources were found that specifically evaluated 35S-directed transgene expression in tree pollen relative to other tree tissues, but expression patterns of this promoter based on other plants should be informative for general comparisons to trees. Thus the “standard” concentration of O_xO in our experiment is likely the highest that would ever be found in transgenic chestnut pollen, so the high (10X) concentration is extremely unlikely to be found in nature. This could be tested more precisely with transgenic pollen if/when larger transgenic trees are allowed to openly form flowers in field conditions.

Daily observations consisted of counting live bees, assessing pollen consumption (mg/day) and replenishing if necessary, visually assessing remaining sucrose solution and replenishing if necessary, counting total constructed nectar & egg cells, and removing any dead bees. Pollen was replenished (filled up to 0.2 g) whenever remaining quantity fell below 0.07 g. A separate pollen cup of untreated pollen was kept near the microcolonies, emptied and refilled every 6 days, and massed daily to quantify mass lost due to evaporation, which was accordingly figured in to all pollen use calculations. Daily pollen use was calculated per individual bee, rather than per whole microcolony, to account for individual bee mortality.

We used linear mixed effect models using the `lme` function in the `nlme` package in R (R Core Team, 2008) to test for significant differences between microcolonies (pollen consumed, cells formed, offspring) receiving none, standard, or high doses of OxO. The source hive from which the microcolonies were generated was set as the random effect for each model. We also generated survival plots using the functions `survfit`, `ggsurvplot`, `survdiff`, and `pairwise_survdiff` in the `survminer` package (Kassambara and Kosinski, 2017), and Cox hazard ratio using the `forest_model` function in the `forestmodel` package (Kennedy, 2017).

One clear result from this study is that bees performed and behaved very differently according to their original source hive. In comparison to source hive effects, pollen treatment had a relatively small impact on survival or behavior. Regardless of source hive, bees in colonies exposed to pollen with the standard concentration of OxO performed similarly to controls without OxO: no significant differences were seen in terms of bee survival, size, daily pollen use, hive construction activity, or reproduction. Figure 9.1.4b shows a Cox Hazard Ratio, indicating the relative hazard of various treatments to bee survival. There are no significant differences in bee survival based on pollen treatment, but source hive (A, B, or C) was a significant factor (mortality was higher in bees from source hives B and C compared to A). Figure 9.1.4c shows mean use of each pollen type over 7 weeks, in 5-day blocks. Again, there are no statistically significant differences by pollen type, though there is a trend near the end of the study toward increased use of pollen with the standard concentration of OxO and decreased use of high-OxO pollen. Table 9.1.4a shows reproductive output: as with survival, there was a significant impact of source hive on reproduction (not shown), but no significant differences ($p > 0.05$) between pollen treatments and the non-OxO control.

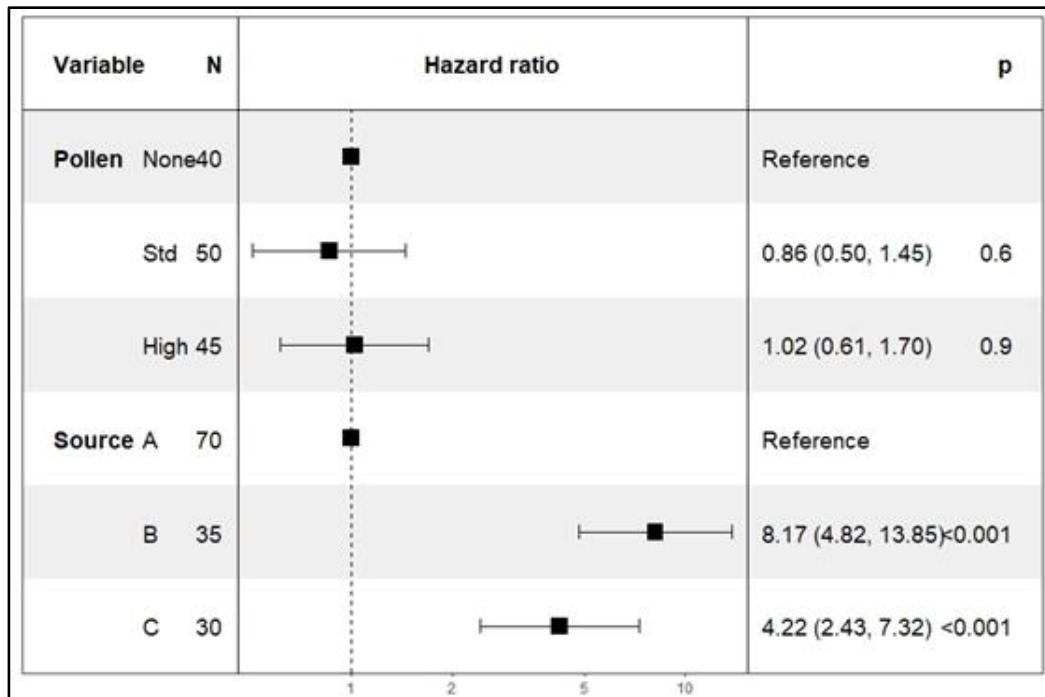


Figure 9.1.4b. Forest plot of Cox proportional hazard model contrasting relative bumble bee mortality (i.e., hazard ratio) by pollen treatment and source hive. “Pollen” indicates OxO treatment concentration (i.e. “None” indicates non-OxO treatment); “Source” (A, B, and C) indicates source colony (hive). Hazard ratio values greater than 1.0 indicate an increased mortality risk relative to reference conditions (non-OxO pollen or Source colony A).

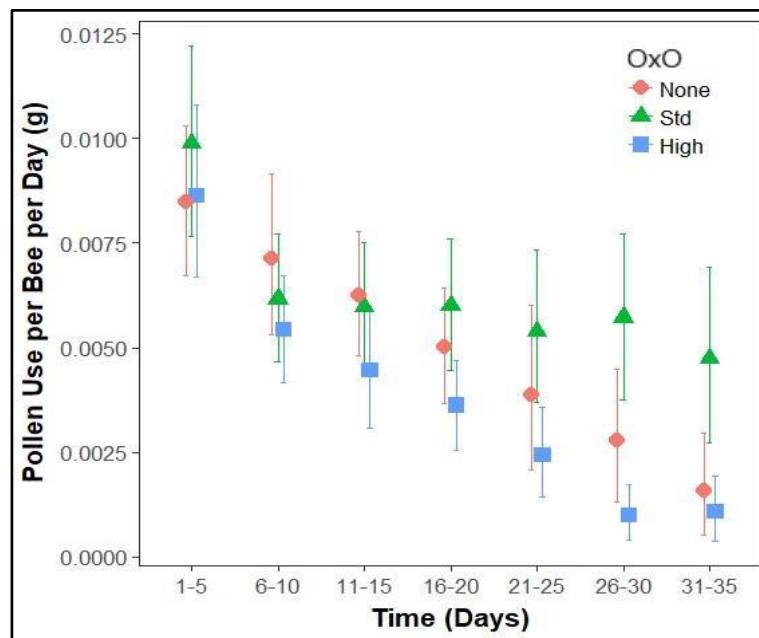


Figure 9.1.4c. Mean pollen use over 5 weeks, in 5-day blocks, by OxO treatment concentration. Error bars indicate 95% confidence intervals.

Table 9.1.4a. Reproductive output of bumble bees by pollen treatment. Presented as mean individuals per microcolony \pm 1 standard error of the mean. Pr > F indicates p value for comparison vs. No OxO treatment, based on Waller-Duncan K-ratio t-test. None of the comparisons between OxO and No OxO treatments were statistically significant ($p > .05$).

| | Eggs | Pr > F | Larvae | Pr > F | Emerged Adults | Pr > F | Combined Offspring | Pr > F |
|----------------|-----------------|--------|-----------------|--------|-----------------|--------|--------------------|--------|
| Std OxO | 4.60 \pm 2.02 | 0.781 | 2.40 \pm 1.06 | 0.910 | 0.30 \pm 0.15 | 0.491 | 7.30 \pm 2.95 | 0.926 |
| High OxO | 1.78 \pm 0.83 | 0.235 | 0.78 \pm 0.47 | 0.575 | 0 \pm 0 | 0.633 | 2.56 \pm 1.25 | 0.268 |
| No OxO Control | 4.75 \pm 1.76 | n/a | 2.13 \pm 1.72 | n/a | 0.13 \pm 0.13 | n/a | 7.00 \pm 3.13 | n/a |

Bees exposed to pollen with the artificially high OxO concentration showed similar survival to the other groups, but showed non-significant trends toward decreased pollen usage and reproduction compared to non-OxO controls. Similar effects are seen when unrealistically high concentrations of natural plant defense compounds or botanical insecticides are supplied to bees: these compounds are often benign or even beneficial to bees at realistic concentrations found in nature, but can have detrimental effects at higher concentrations (Manson and Thomson, 2009; Manson *et al.*, 2010; Köhler *et al.*, 2012; Cook *et al.*, 2013; Arnold *et al.*, 2014; Xavier *et al.*, 2015; Stevenson *et al.*, 2017). While oxalate oxidase itself doesn't have known harmful effects against living organisms, the hydrogen peroxide byproduct can have antimicrobial properties (Baldry 1983) or sub-lethal effects on insects (Ramputh *et al.*, 2002). However, production of high concentrations of hydrogen peroxide seems unlikely in pollen based on low 35S transgene expression in these cells and presumably limited quantities of the oxalate substrate required for oxalate oxidase activity. Even if these trends of effects at the artificially high OxO concentration are biologically significant, when taken in context of observations on secondary metabolite effects on bees, this is not an unusual pattern with natural plant defense compounds. Additionally, it is not realistic that bees would ever be exposed to this high-OxO concentration in field conditions, even if they foraged exclusively on transgenic chestnut pollen. In summary, a realistic concentration of oxalate oxidase in chestnut pollen does not present novel risks to bumble bees.

9.2 Responses to other pests and environmental stresses

Apart from chestnut blight tolerance, no differences between transgenic and non-transgenic trees have been observed in response to plant-disease, plant-arthropod, or plant-environment interactions. Apart from the *Phytophthora* study described below, targeted pest surveys have not been conducted on Darling 58 or offspring lines, but first-hand observations based on our 12 years' experience growing transgenic chestnuts are reported here. During acclimatization from tissue culture, fungal damage to the shoot tip is a common scenario when the plantlets are young, soft, and maintained in a humid environment. This is due to environmental fungal contaminants,

which are not pathogenic on chestnuts outside of this artificial growth environment. We consistently fail to observe notable differences between transgenic and non-transgenic American chestnuts as a result of this type of fungal infection; both tree types can be infected by environmental fungal contaminants.

Transgenic and non-transgenic chestnuts in greenhouse conditions do not appear differentially susceptible to damage from common greenhouse pests such as mealybugs, spider mites, and powdery mildew. This was specifically observed by research collaborators in preparation for a *Phytophthora* screening experiment in 2018 (Appendix I). This experiment consisted of Ellis, Darling 58, and various other transgenic tree types that had been transported to the US Forest Service Resistance Screening Center in North Carolina. Before the screening experiment began, low-level pest infestations including powdery mildew and spider mites became dramatically worse in the new greenhouse conditions, completely infecting all transgenic and non-transgenic trees that had been transported. These are common generalist greenhouse pests, unrelated to blight or *Phytophthora*, that were reported by greenhouse staff but not identified to species. All of the trees in this experiment had to be devitalized immediately after the infestation was observed in order to protect other plants in the greenhouse.

A similar *Phytophthora* screening experiment on Darling 58 offspring was conducted in 2019, this time without incidental pest problems. *Phytophthora cinnamomi* is especially relevant to American chestnuts as it is a major pest in the southern part of the chestnut's natural range (Wang *et al.*, 2013). This study was performed at the USDA Forest Service Southern Region Resistance Screening Center, which regularly screens trees for *Phytophthora* susceptibility. Tree types in this study included transgenic T2 seedlings and non-transgenic full siblings from the same crosses, sent to the Screening Center in spring 2019 (Appendix I). All tree types were exposed to two strains of *Phytophthora cinnamomi* in early summer, and over the following twenty weeks, nearly all T2 seedlings (both transgenic and non-transgenic) succumbed to *Phytophthora*. Results are described in a summary report from the Resistance Screening Center manager (McKeever, 2019). We conclude from this study that presence or expression of OxO in Darling 58 offspring does not affect susceptibility to *Phytophthora*, further confirming a lack of plant-pest interactions outside of blight tolerance.

Controlled experiments with insect herbivores have been conducted on Darling 58 (Section 9.1.3) and other OxO-expressing chestnut events (Section 10.6.7). Field-grown chestnuts have also been observed to sustain incidental insect herbivory, powdery mildew infections, and rodent chewing, regardless of transgene presence. While challenges by these incidental pests have not been intentionally applied or quantified, non-transgenic controls and Darling 58 American chestnuts have been impacted by these pests at apparently similar rates. These observations on Darling 58 chestnuts at various growth stages, along with experimental results in Sections 9.1 and 10.6, confirm that presence of OxO does not affect environmental interactions with other organisms apart from chestnut blight tolerance, and we have no evidence that planting of Darling 58 chestnuts would affect incidence of other pests or pathogens any more than planting traditionally bred chestnuts.

Similarly, drought stress or overwatering have occasionally been unintentionally applied during growth chamber, greenhouse, and outdoor care of chestnuts, but we have not observed Darling

58 offspring to respond any differently than non-transgenic relatives to these stresses. Intentional experiments have not been conducted on Darling 58 regarding abiotic stress tolerance, but numerous anecdotal observations on combined batches of transgenic and non-transgenic chestnuts have not shown obvious differences. The most likely mechanism for abiotic stress tolerance differences due to OxO is production of hydrogen peroxide (Singh *et al.*, 2006; Wan *et al.*, 2009; Section 4.5.2). As discussed in Sections 4.3 and 6.3.2, increased production of H₂O₂ in Darling 58 is logically restricted to cankers, where oxalate is supplied by the blight fungus. Non-transgenic trees also produce hydrogen peroxide in response to infection (Section 6.3), so abiotic stresses should not result in substantial changes compared to non-transgenic chestnuts. Additionally, meaningful testing for abiotic stress tolerance has been reported to be difficult in trees, and not necessarily consistent between greenhouse and field conditions (Euliss *et al.*, 2008; Brunetti *et al.*, 2011), which would further complicate potential comparisons between transgenic and non-transgenic trees.

9.3 Potential spread of OxO to other *Castanea* species

The only logical means by which transgenes from Darling 58 could spread to related species is through inheritance by viable offspring from successful pollination with at least one transgenic parent. American chestnut is capable of hybridizing with other members of the genus *Castanea*, either through controlled pollination or open pollination, though differential timing of flower development may represent one possible barrier to open pollination. Interspecific hybrids are often male-sterile (unable to produce viable pollen; Section 2.1.2), but are capable of setting fruit if female flowers are pollinated by a compatible donor (Anagnostakis, 2012; Sisco *et al.*, 2014). Darling 58 chestnuts have produced offspring through controlled pollinations with *C. dentata* x *C. mollissima* F1 hybrids, Allegheny chinquapin, and European chestnut (unpublished data). These offspring generally appear healthy but are not yet of flowering age, so we have no data regarding rates of male sterility or seed production in subsequent generations. No Darling 58 trees are yet mature enough to produce female flowers, and any male flowers have been either bagged or produced indoors to comply with APHIS permit regulations, so we have no data on natural pollination rates for Darling 58 trees.

Though many more years of research will be required to produce data about interspecific hybridization of Darling 58 and compatible species, we have no reason to expect that pollen viability, fertilization rates, or any other aspect of sexual reproduction would differ between Darling 58 American chestnut and non-transgenic American chestnut. Male sterility in hybrid offspring appears to be a result of interactions involving cytoplasmic (chloroplast or mitochondrial) genes in American chestnut (Sisco *et al.*, 2014; Section 2.1.2), so the presence of transgenes in the nuclear genome of Darling 58 should not change these interactions. Therefore, we expect that Darling 58 trees growing in close proximity (Section 2.6.2, Section 11.6) to other *Castanea* species could produce viable hybrid offspring. Historically, American chestnut was reported to hybridize with chinquapin species where their ranges overlapped (Section 2.1.1). Hybrid offspring inheriting the OxO gene would be expected to exhibit blight tolerance. Given the expected slow rate of chestnut reproduction and spread (Section 2.2.2), and limited effective pollination distances (Section 2.6.2), such natural hybridization events would be rare, especially

while mature Darling 58 trees and their offspring are uncommon, a time period of several decades to several centuries following the introduction of Darling 58 to the landscape (Section 11.6).

Plant breeders may intentionally create hybrid chestnut varieties for agricultural or horticultural use, incorporating the OxO gene for blight tolerance. This could also be achieved through separate transformations directly into other *Castanea* backgrounds rather than by hybrid breeding, but given the presence of other native and non-native *Castanea* species in and near the range of *C. dentata*, the possibility of natural introgression exists. Depending on blight susceptibility and goals of land managers, occasional natural introgression of the trait into other chestnut species would not necessarily be met with opposition (Section 11.4). We have not seen any detrimental effects on human health, non-target organisms, or nut quality in transgenic American chestnuts (Sections 8 through 10), so it is unlikely that the inclusion of OxO would have a detrimental effect on other *Castanea* species or hybrids, or other organisms in their respective environments.

No hybridizations between American chestnut and any other Fagaceae outside of genus *Castanea* have been documented, so gene travel outside the genus is not a realistic concern. Horizontal gene transfer, which can occur if microbes incorporate plant genes into their own genomes, is also theoretically possible. However, horizontal transfer of oxalate oxidase is already possible via the many other endogenous sources of this gene as described in Section 4.2, so Darling 58 would not be a unique source of this gene in the environment.

9.4 Persistence of OxO activity in Darling 58 leaves

Persistence of transgene products in shed leaves is one potential concern that has been explored regarding environmental interactions with transgenic plants (Sun *et al.*, 2007; Winder *et al.*, 2013). To test this with transgenic chestnuts, mature leaves were removed from late-season Darling 58 plants after they had set dormant buds. Leaves at this point were relatively tough and very dark green, but still pliable and not yet yellowing. Discs (6mm diameter) were punched from each leaf and screened with an OxO histochemical assay (Section 7.4.1). Screening started immediately before leaf removal and continued every other day thereafter, until a given leaf showed no more OxO activity. Ablated leaves were stored in a cool greenhouse (8 – 10°C day, 3 – 5°C night) used for maintaining chestnut trees in a dormant state. A young leaf from an actively growing greenhouse plant and an attached leaf from a late-season plant were used as positive OxO activity controls; discs were removed and screened from attached leaves on these plants at the same times as the ablated leaves.

Discs from attached leaves on both control plants consistently showed OxO activity (distinct black ring, Figure 7.4.1b) throughout the course of each experiment (11 days). OxO activity in ablated leaves was detectable for at least 5 days, but entirely stopped as soon as a leaf dried enough to begin to curl (Figure 9.4a). Leaf drying (and cessation of OxO activity) occurred after approximately 8 days (range 6 – 11 days) in these conditions. We acknowledge that leaf desiccation *in situ* may vary depending on ambient conditions, and under certain artificial conditions OxO activity can be prolonged for much longer (Section 10.6.4). However, the general principle of OxO activity ceasing upon leaf drying should be broadly applicable. This was also visualized by testing an excised strip of tissue from an older, senescing leaf on a dormant plant: this strip extended from green/living tissue in the center of the leaf to brown/dead tissue at the

edge of the leaf. Again, OxO is active in green/active tissues, but not in brown/dead tissues (Figure 9.4b). Overall results were similar to a previous experiment performed on Darling 4 leaves (Section 10.6.4). The similarity of results between transgenic lines with different expression levels suggests that overall OxO transcript levels in active plants don't substantially affect the persistence of OxO activity in desiccating/inactive tissues.



Figure 9.4a. Curled/dry leaf (bottom), showing state in which OxO is no longer active, compared to fresh leaf (top). Dry leaf was repeatedly tested with an OxO histochemical assay (hole punches visible) until OxO activity ceased (approximately 1 week).

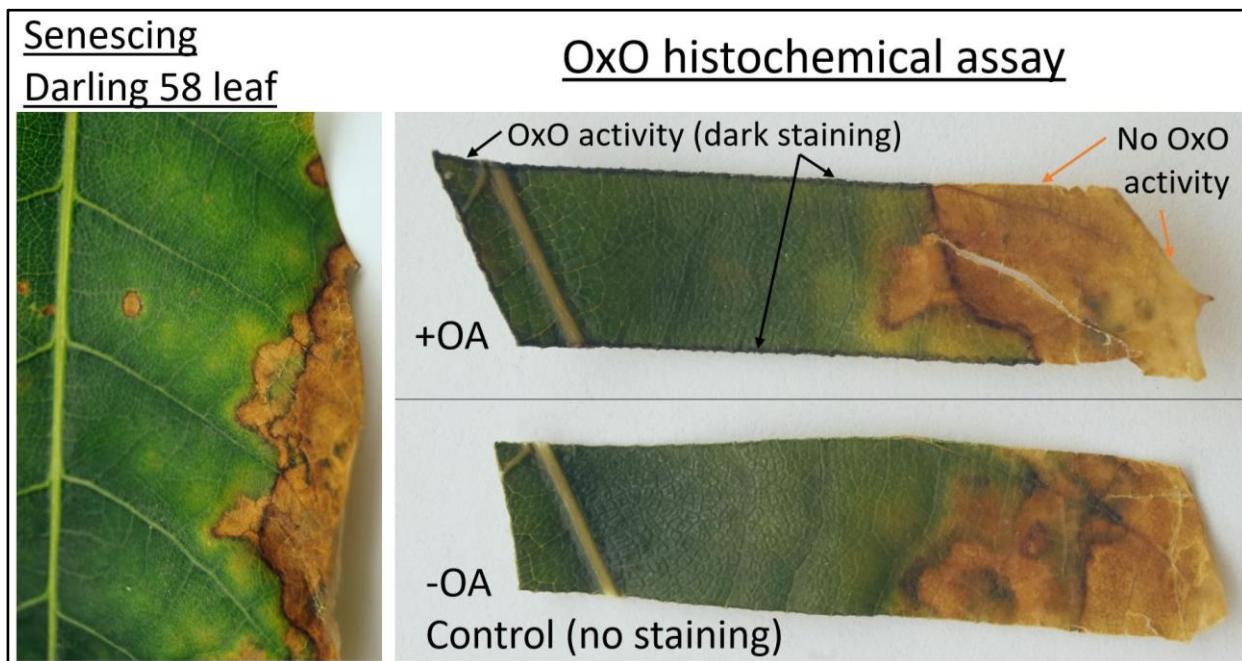


Figure 9.4b. Partially senesced leaf, with excised strips showing OxO activity at the edges of living/green portions (black arrows), and a lack of OxO activity in dry/dead portions (orange arrows).

9.5 Potential effects of blight-tolerant chestnuts on *C. parasitica*

We do not yet have experimental data on infection and reproduction rates of the chestnut blight fungus on large Darling 58 trees in a forest setting, but a long-term experiment has been initiated that will provide data on these questions in the years to come (see BRAG project description, Section 11.2). The following is a logical exploration of potential effects of restoration with a blight-tolerant American chestnut such as Darling 58. Related topics are discussed in Sections 5.3, 5.4, 6.3, and 6.4.

The chestnut blight fungus is capable of colonizing and reproducing on Darling 58 trees without causing fatal cankers (Section 8.1), as it does on Chinese chestnut and many species of oaks. In other words, blight-tolerant host trees can survive despite infections of *C. parasitica*, allowing the fungus to persist, while blight-susceptible trees eventually die back. Dying chestnut trees or dead stems temporarily serve as hosts to the blight fungus while it survives as a saprophyte (Prospero *et al.*, 2006), but ultimately, mortality in a population of entirely disease-susceptible host trees reduces suitable host material for a pathogen. In contrast, Darling 58 can indefinitely serve as a host for the blight fungus. Furthermore, reproductively mature Darling 58 trees will continually produce non-transgenic, blight-susceptible offspring (Section 6.4). Thus it is likely that potential chestnut restoration scenarios including blight-tolerant host trees would not be detrimental to the blight fungus, allowing it to persist by increasing the longevity and/or prevalence of host trees.

In the context of alternative blight management or restoration strategies, it is likely that any breeding program involving Asian chestnuts would be similarly benign in terms of effects on the blight fungus. *C. parasitica* has apparently persisted for millennia in its native range of Asia where host chestnuts are tolerant or resistant to infection, so restoration scenarios with blight-tolerant trees in the US could result in similarly stable relationships (Section 6.3.2), with a corresponding lack of novel plant pest risks. In contrast, hypoviruses (Section 3.3.1) can directly modify the blight fungus, and result in slowed growth and reduced sporulation (Rigling and Prospero, 2018). It is worth noting that hypovirulence can apparently occur naturally (Milgroom and Cortesi, 2004), that not all spores produced by hypovirulent fungi are infected with the virus, and that intentional applications of hypovirulent fungi may be regulated or restricted in some areas.

10.0 Legacy transgenic American chestnut events

Prior to development of the Darling 58 event, which we are submitting for consideration of nonregulated status, we produced several other OxO-expressing transgenic American chestnut lines. Many of these were also designated Darling lines because they contain the OxO gene, but only the Darling 58 event is currently being submitted for consideration of nonregulated status. Several of these “legacy events” are mentioned and described in this petition for general comparisons, because they have been included in relevant experiments or publications. However, only a few are intended to be used directly as bridging data comparators with Darling 58, as shown in Table 1.3a and explained in Section 10.2. This section will clarify background details, characterization, and nomenclature of Darling 4 and other relevant events, including rationale for the use of bridging data from selected events, with a focus on how these legacy events compare to Darling 58 (Tables 1.3a and 10.1a).

10.1 Background on other OxO-expressing transgenic events

The first OxO-containing transgenic chestnut event that we propagated, tested, and planted outdoors is known as Wirsig. This event contains the pΔVspB-OxO vector (Polin *et al.*, 2006), which contains OxO controlled by the vascular-directed vegetative storage protein (vspB) promoter from soybean, as well as a green fluorescent protein (mGFP5-er) marker gene in the same vector, which is constitutively controlled by the CaMV-35S promoter and terminator (see Table 10.1a for transgene and vector components in all events). The vspB promoter in this particular construct was eventually found to have a 726 bp deletion, which resulted in reduced OxO expression in the Wirsig line, so this event did not have desired levels of blight tolerance. Wirsig resulted from a transformation into the background American chestnut tissue line known as WB275-27 (Table 10.1a), though the name for this isogenic line has been informally shortened to WB275 in some experiments and previous publications.

We subsequently replaced the deletion in the vspB promoter to optimize OxO expression, and also transitioned to a co-transformation procedure, using separate vector constructs for the gene of interest (GOI, e.g. OxO) and the visual marker gene (GFP). Darling 1, Darling 4 (Newhouse *et al.*, 2014b) and Darling 5 are examples of co-transformed American chestnut events containing vectors pGFP and pTACF3, again with OxO controlled by the improved vascular vspB promoter (Table 10.1a, Figure 10.4.4a). Full T-DNA sequence data from the pTACF3 vector are shown in Appendix IX. The pGFP construct contains the green fluorescent protein gene controlled by the CaMV-35S promoter and terminator, and a phosphinothricin acetyltransferase (BAR) selectable marker for controlled by the Ubi-3 promoter and terminator. Darling 4 has two copies of each construct, while Darling 5 has one of each (Table 1.3a). AN2G3 is a visual marker-only transgenic event in the same genetic background as Darling 1, 4, and 5, but containing only pGFP, without a separate gene of interest. All of these events are in the same WB275-27 background line as Wirsig. Darling 4 showed visibly enhanced blight tolerance compared to wild-type American chestnut (Section 10.5.1), but less than the naturally blight-tolerant Chinese chestnut (Newhouse *et al.*, 2014b).

*Table 10.1a. Background details and construct components of selected chestnut transgenic events. GOI = Gene of Interest (primary transgene), Term. = terminator. *Darling 58 is the only event being submitted for consideration of nonregulated status. “ΔvspB (partial)” refers to a partial deletion eventually detected in the promoter in Wirsig; see description in Section 10.1.*

| Events: | Wirsig (Polin <i>et al.</i> , 2006) | Darling 1, 4, 5 (AN2G3 = pGFP only) (Newhouse <i>et al.</i> , 2014b) | Darling 11, 215, 311 (Zhang, B. <i>et al.</i> , 2013) | Darling 54, 58* (Sections 6 - 10) |
|---|--|--|---|--------------------------------------|
| Element | | | | |
| Background Genotype | WB275-27 | | Ellis | |
| Year embryo culture established <i>in vitro</i> | 2000 | | 2007 | |
| Parent Tree | WB275 | | Pond #1 | |
| Parent Tree Location | Meadowview, VA | | Windsor, NY | |
| Year Transformed | 2004 | 2007 | 2011 | 2012 |
| Vector Construct | pvspB-OxO | pTACF3 | p35S-OxO | p35S-OXO |
| GOI Promoter | ΔvspB (partial) | vspB | CaMV-35S | CaMV-35S |
| Gene of Interest (GOI) | OxO | OxO | OxO | OxO |
| GOI Terminator | Act2 | Act2 | Act2 | Act2 |
| Selectable Marker Promoter | Ubi-3 Promoter | UBQ10 | UBQ10 | UBQ10 |
| Selectable Marker Gene | BAR | NPTII | NPTII | NPTII |
| Selectable Marker Terminator | Ubi-3 Term. | Nos | Nos | Nos |
| Visual Marker Promoter | CaMV-35S | N/A (Visual marker on separate construct) | N/A (Visual marker on separate construct) | N/A (No visual marker) |
| Visual Marker Gene | GFP | | | |
| Visual Marker Terminator | 35S Term. | | | |
| 2nd Vector Construct (co-transformed) | N/A (Not co-transformed) | pGFP | pGFP | N/A (Not co-transformed) |
| Selectable Marker Promoter | | Ubi-3 Promoter | Ubi-3 Promoter | |
| Selectable Marker Gene | | BAR | BAR | |
| Selectable Marker Terminator | | Ubi-3 Term. | Ubi-3 Term. | |
| Visual Marker Promoter | | CaMV-35S | CaMV-35S | |
| Visual Marker Gene | | GFP | GFP | |
| Visual Marker Terminator | | 35S Term. | 35S Term. | |

Around the same time as Darling 4, co-transformations were done with pGFP and pTACF7 (Newhouse *et al.*, 2014b), which contained the *vspB*-controlled OxO as well as another GOI encoding a synthetic antimicrobial peptide called ESF39 (Powell *et al.*, 2000), controlled by the ACS2 vascular-directed promoter (Connors *et al.*, 2002). These co-transformed dual-GOI events were called Hinchee 1 and Hinchee 2. This antimicrobial peptide had previously shown promise for enhancing resistance to fungal diseases in trees (Liang *et al.*, 2002; Newhouse *et al.*, 2007), but at least as expressed in these American chestnut events, these genes in combination did not appear to further enhance blight resistance beyond Darling 4 (OxO alone). Additionally, we acknowledge that a transgene encoding a broad-spectrum antimicrobial compound would likely introduce additional complexities with non-target microbes, so ecological comparisons would have to be considered especially carefully if trees containing such a gene were intended for wild release. Hinchee events are included in some studies described in this petition, but they are not intended for direct comparison or bridging data with Darling 58.

In order to further enhance blight tolerance in transgenic American chestnuts, we used the constitutive CaMV-35S promoter in the p35S-OxO vector, as described in Section 7.1. We also transitioned to a different background line called Ellis (sometimes called Ellis 1), which is native to New York. Some of the earlier events produced with the p35S-OxO vector (including Darling 11, Darling 215, and Darling 311) showed significantly enhanced blight tolerance approaching or surpassing that in Chinese chestnut controls (Figure 10.5.1d; Zhang, B. *et al.*, 2013), but they were still co-transformed with a vector containing GFP. In order to minimize the total number of transgenes and eliminate unnecessary visual marker genes, subsequent transformations were done without GFP, resulting in events including Darling 54 and Darling 58. Both these events show good blight tolerance and their offspring show approximately expected inheritance patterns (Sections 6.4 and 10.3). However, the transgene insertion site in Darling 54 event was subsequently determined to be inside an intron of a predicted native gene (Section 10.4.2), so we removed this event from regulatory consideration. The transgene insertion site of Darling 58 does not interrupt an existing gene or alter expression of nearby genes (Section 7.2.3).

Various analyses have been performed on the OxO-expressing transgenic lines described above, many of which are included in this petition to support or clarify Darling 58 data. Table 1.3a shows what data are presented for each relevant bridging event, the source(s) of transgenic tissues or plants for each experiment, and where in this petition the data are presented.

10.2 Rationale for the use of legacy events for bridging data

Many non-target experiments and other tests have been done on older transgenic lines than Darling 58. The age and size of trees from these older lines, especially Darling 4, have allowed experiments not possible with more recently generated Darling 58 material (Sections 10.5, 10.6). Darling 4 demonstrated enhanced blight tolerance (Newhouse *et al.*, 2014b) and contains the same OxO transgene as Darling 58. But OxO in Darling 4 is controlled by a nominally vascular promoter (*vspB*) instead of a constitutive promoter (35S), and thus is expressed primarily in the vascular tissues and at a lower overall level. While 35S transgene products would be found more prevalently in additional tissues compared to those from *vspB*, spatial patterns of OxO reaction byproducts such as H₂O₂ would likely be similar. This is because the oxalic acid substrate for the OxO reaction is only present near cankers, which form primarily in or near vascular tissues. In other words, while expression of OxO is higher in leaves from Darling 58 than Darling 4 (Figure 7.3b), enzymatic activity of OxO and generation of byproducts in leaves would likely be similar between these two events (unless there was another source of oxalic acid.)

Additional rationale for using bridging data from Darling 4 applied to Darling 58 is that Darling 4 contains multiple copies of multiple vector constructs, with two additional constitutively expressed transgenes (Tables 1.3a and 10.1a), and therefore should have a greater chance of experiencing unexpected insertion effects than Darling 58. Insert location of the various vector constructs was not identified in Darling 4, so we do not know whether native genes are disrupted in this event: again, any potential changes in Darling 4 are likely the same or more severe than those in Darling 58. As a result, if no adverse effects are seen with Darling 4 due to the genetic engineering techniques or insertions, it is unlikely that Darling 58 (with fewer total changes) would have any adverse effects. Limitations of using Darling 4 compared to Darling 58 include lower overall levels of OxO expression, different spatial patterns of OxO expression, different insert locations, and different genetic backgrounds (WB275-27 vs. Ellis 1; Table 10.1a). Possible effects of the insert location and OxO expression in Darling 58 are covered elsewhere in this petition (Sections 6 and 7).

Other legacy events have been included for specific comparisons with Darling 58, but not for broader analyses or impacts. For example, Darling 5 is used as a comparator for Darling 58 copy number using qPCR, since Southern analyses concluded that Darling 5 has one copy of the OxO construct (Sections 7.2.2 and 10.4.1). However, other characterizations such as relative transgene expression, phenotypic comparisons, or environmental interactions are not presented for Darling 5, as they are not relevant to copy number comparisons. Likewise, Darling 215 was previously established as a comparator for blight tolerance and mRNA expression analyses by RT-qPCR (Zhang, B. *et al.*, 2013), so it is presented in that context here (Section 7.3), but other data such as insert location are not essential to understanding the mRNA expression comparisons.

One other legacy event, Darling 54, was produced at the same time as Darling 58 and has the same genetic background (Ellis 1), same complement of transgenes (single copy of p35S-OxO), and similar OxO expression levels (Sections 10.4.3 and 10.4.4), so the genomic insertion site is essentially the only difference. Darling 54 and T1 offspring were used for some experiments along with Darling 58, but we recently determined that the insertion site in Darling 54 lies within an intron of a predicted gene in the chestnut genome (Section 10.4.2), so this event is not being

submitted for regulatory consideration. Experimental data from Darling 54, however, are still useful to assess whether OxO expression and/or transgene insertion affects the phenotype of the plant or its interactions with other organisms (Sections 10.5, 10.6).

If differences in phenotype, environmental interactions, or plant pest risks exist due to genomic changes (Kumar and Fladung, 2002) or the presence of the OxO transgene, we would expect to see them in Darling 4 and 54 at least as much as in Darling 58. Therefore these and other events are useful for bridging data comparisons with Darling 58, despite their individual differences.

10.3 Transgene inheritance by offspring of legacy events

Controlled pollinations with Darling 4 pollen were performed between 2012 and 2015, resulting in a combined total harvest of 140 Darling 4 T1 nuts. Of these, 51 were transgenic (~36%). This < 50% inheritance rate likely reflects unintended open pollination due to inefficient pre-bagging of female flowers before controlled pollination (Section 6.4), especially since a multi-copy parent like Darling 4 (Section 10.4.1) could theoretically result in more than half of the offspring inheriting at least one copy of the transgene (unless the transgenes were inserted in tandem or on the same chromosome).

Pollen from other OxO-transformed lines (Section 10.1) was used for limited numbers of pollinations from 2011 to 2016. Proportions of transgenic offspring vary from 29% to 80% in these small sample sizes, but overall combined inheritance rates are close to the expected 50% from a single-copy transgenic parent: Darling 54 (2 viable transgenic nuts of 7 total), Darling 311 (4 transgenic of 5 total), Darling 11 (7 transgenic of 12 total), and Wirsig (16 transgenic of 24 total).

10.4 Molecular characterization of legacy events

Whole genome sequencing is in progress for Darling 4 and the isogenic line WB275-27, which should further clarify details regarding insert location, copy number, structure, etc. Results will be shared when they are available (anticipated in 2020). In the meantime, this subsection describes data that are currently available on Darling 4 and other events used for bridging to Darling 58.

10.4.1 Characterization of transgene copy number in legacy events

Portions of this section are excerpts from Newhouse *et al.* (2014b).

Southern hybridization is a traditional method of confirming transgene presence and copy number, distinguishing unique events, and elucidating insert structure. All Southern experiments were derived from leaf tissue collected from > 6-month-old individual trees of confirmed transgenic lines, each of which had originated from a single tissue culture shoot (Section 6.2.2). Genomic DNA for Southern hybridization on transgenic chestnuts was extracted with the “Carlson/Qiagen Method” (Csaikl *et al.*, 1998), and digested separately with restriction enzymes *Eco*RI and *Xba*I (each cuts only once in the T-DNA of each construct, Figure 10.4.1a and Appendix IX. The use of two separate restriction enzymes enhances accuracy by reducing the chance that closely spaced transgene copies will appear to be a single insert.

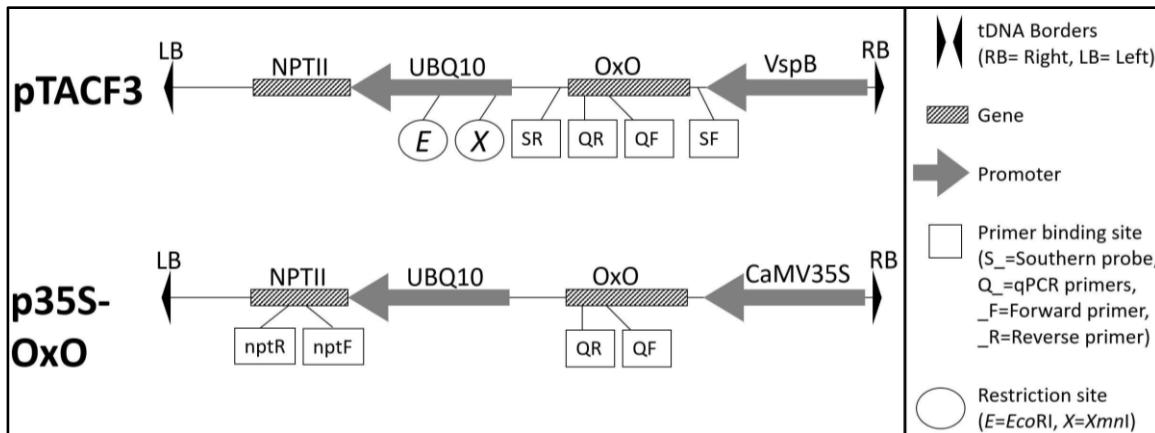


Figure 10.4.1a. Schematic transgene vector maps comparing pTACF3 (used in Darling 4 and 5) with p35S-OxO (used in Darling 58), including details relevant to copy number analysis by Southern hybridization and qPCR. See Section 7 for more details on Darling 58 transgenes and Section 10.1 for further detail on legacy events.

Digested DNA was separated on a 0.8% agarose gel at 48 V, and blotted to a positively charged nylon membrane (Amersham Hybond –N+, Buckinghamshire, UK) with the “Genie” electrophoretic blotter (Idea Scientific, Minneapolis, MN). A 966 bp probe (Table 7.2.1a) was designed to contain the coding region of the OxO gene (Figure 6.1.2a), and labeled with 32P-dCTP (Stratagene Prime-It II Random Primer Kit). Hybridization was performed in Stratagene QuikHyb buffer (Agilent, Santa Clara, CA) for 2 hours at 65°C, and washed according to Stratagene’s instructions. The blot was exposed to Kodak BioMax MS film for 12 – 18 hours at -80°C, and developed with Kodak GBX film developing solutions. Films were digitized by grayscale scanning on a Canon CanoScan LiDE 210 flatbed scanner, cropped to fit, and lanes were numbered sequentially; no further image editing was done (Figures 10.4.1b and 10.4.1c).

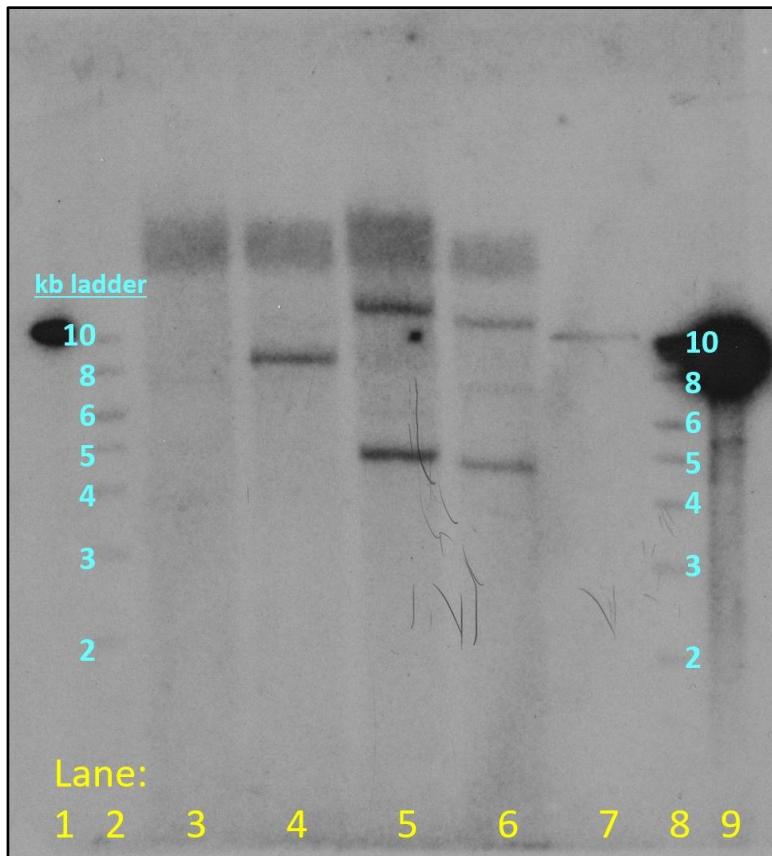
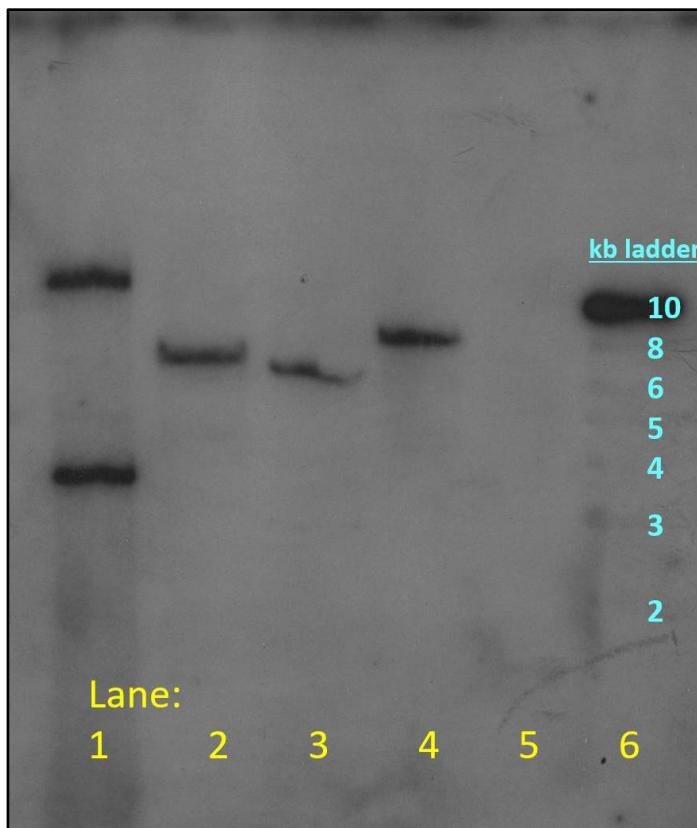


Figure 10.4.1b. Southern hybridization film from 5/12/2009, including EcoRI digests of legacy events Darling 4 and Darling 5, hybridized with a radio-labeled OxO probe. Lane contents, from left: 1. Positive control (pTACF7 plasmid, narrow lane), 2. Kilobase ladder (narrow lane; fragment sizes marked in kb with blue numbers), 3. WB275-27 (non-transgenic chestnut control), 4. Darling 5, 5. Darling 4, 6. Darling 4 (separate individual plant), 7. Positive control (low concentration pTACF7), 8. 1 kilobase ladder (narrow lane), 9. Positive control (high concentration pTACF3 plasmid, narrow lane).



*Figure 10.4.1c. Southern Hybridization film from 8/7/2009, including *XmnI* digests of legacy events Darling 4 and Darling 5, hybridized with a radio-labeled Oxo probe. Lane numbers, from left: 1. Darling 4, 2. Darling 5, 3. Wirsig, 4. Wirsig (repeat with separate restriction digest), 5. AN-2G3 (marker-only non-Oxo control), 6. Positive control with size marker (*pTACF7* plasmid plus 1 kilobase ladder; fragment sizes marked in kb with blue numbers).*

According to Southern hybridization with the radio-labeled Oxo probe (Figures 10.4.1b and 10.4.1c), Darling 4 was determined to have two insertions of the Oxo transgene construct (two bands in each lane), and Darling 5 was determined to have a single copy (one band per lane). Results on genomic DNA samples digested with two separate restriction enzymes confirm the result. Thus Darling 5 was used as a known single-copy standard for subsequent copy number comparisons, and Darling 4 was included as a two-copy control (Section 7.2.2). Other transgenic events (e.g. Wirsig and AN-2G3) were also analyzed via Southern hybridization and appear in these films, but were not used for direct comparisons with Darling 58 or subsequent experiments.

Relative copy number on Darling 4 and Darling 5 was also evaluated by qPCR, and Southern hybridization results were used for bridging to Darling 58 (Section 7.2.2).

10.4.2 Characterization of transgene insertion site for Darling 54

Insert location of the Darling 54 event was analyzed with Genome Walking to obtain flanking sequence data similar to Darling 58 (Section 7.2.3). Unlike Darling 58, the insert in Darling 54 was determined to be inside the intron of a predicted gene. Specifically, this predicted gene shows a 98% identity match to a “SAL1 phosphatase” gene from *Quercus suber* (NCBI Gene Symbol LOC112009029), and matched to Chinese chestnut genome scaffold number 04069 (located

between 22,000 – 23,000 bp according to the Hardwood Genomics Project (2019); Staton *et al.*, 2015). There were no visible abnormalities or growth differences in young Darling 54 trees or their T1 offspring due to this insertion, and it is possible that its location in an intron of this gene would not cause any problems. Even so, this event was removed from regulatory consideration due to this insert location in a native gene.

10.4.3 Characterization of transgene mRNA expression by RT-qPCR in legacy events

Expression of OxO mRNA from legacy events was tested via RT-qPCR using similar methods to those described for Darling 58 in Section 7.3. Darling 4 typically shows substantially lower expression than Darling 58, especially in leaf tissues (Figure 10.4.3a). Expression of OxO by Darling 4 is more localized, since it is controlled by the vascular-specific vspB promoter (Section 10.1), so expression and activity are higher in certain tissues (Section 10.4.4) that may not be reflected in mRNA extractions from whole-tissue samples. In other words, the very low OxO expression shown by Darling 4 in Figure 10.4.3a likely underrepresents expression levels in vascular tissues that are particularly relevant to blight, while the 35S promoter in Darling 58 and Darling 311 expresses very highly in leaf tissues (Figure 7.4.2a). Regardless of specific ratios and tissue types used for mRNA studies, Darling 4 clearly expresses OxO at levels adequate to be detectable with activity assays (Section 10.4.4) and provide moderately enhanced blight tolerance (Section 10.5.1).

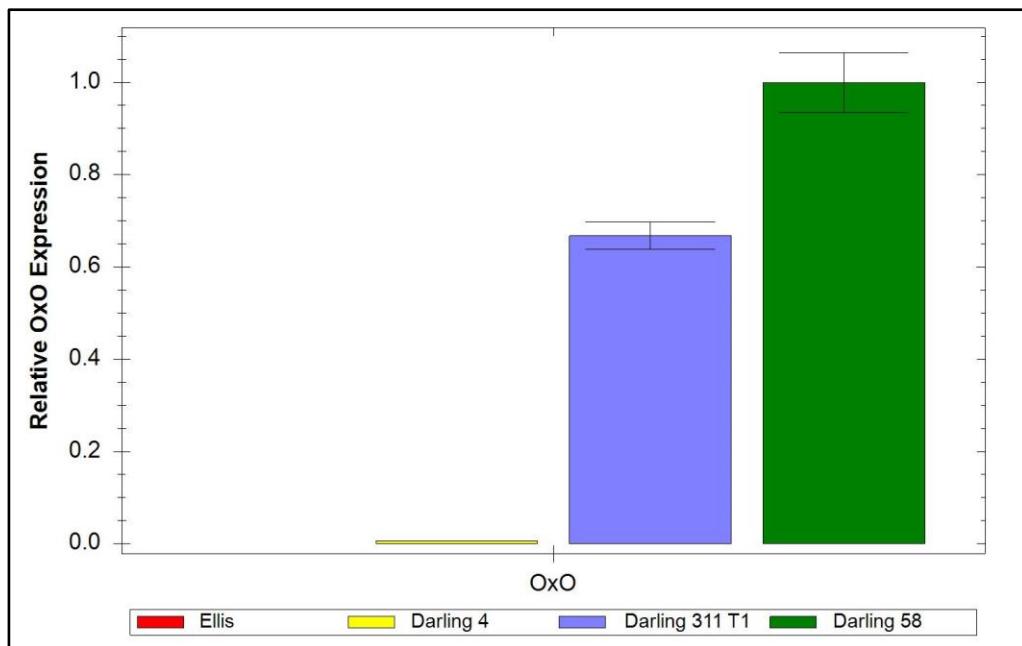


Figure 10.4.3a. Reverse-transcriptase quantitative PCR (RT-qPCR) determination of OxO expression in leaf tissue, including Ellis (no expression), Darling 4, T1 offspring of Darling 311, and Darling 58 for comparison. Reference genes were Actin and ef1. Portions of this figure are repeated from Figure 7.3b. Raw data are shown in Appendix V.

As described in Section 7.3, an event called Darling 215 was established as a minimum OxO expression threshold for adequate blight tolerance in transgenic American chestnuts (Zhang, B. *et al.*, 2013). Darling 54 (Section 10.1) was found to have OxO expression similar to or slightly less than Darling 58, both of which are greater than Darling 215 (Figure 10.4.3b).

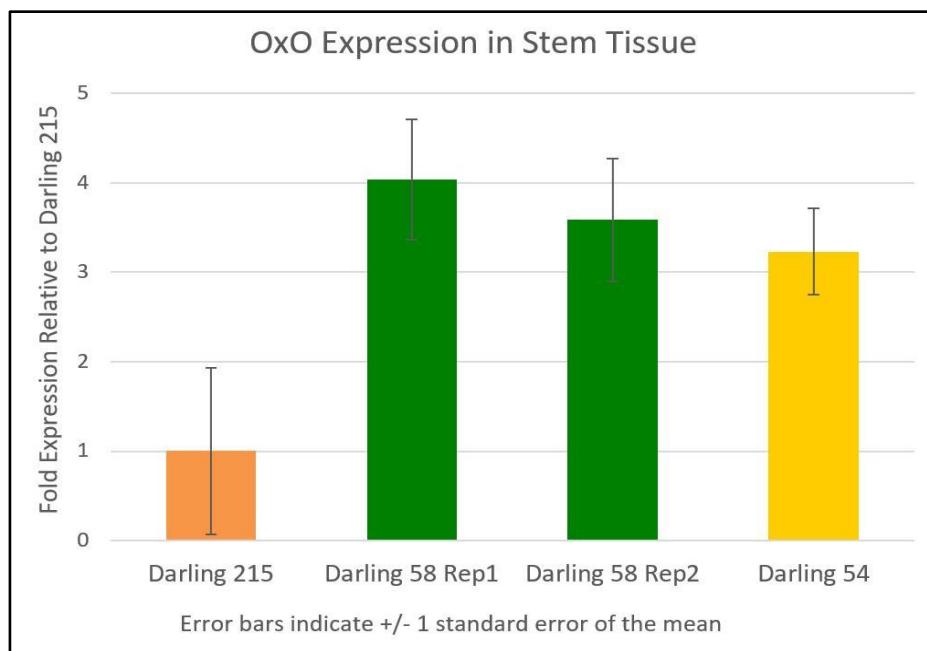


Figure 10.4.3b. Reverse-transcriptase quantitative PCR (RT-qPCR) determination of OxO expression in stem tissue, comparing two replicates of Darling 58 to Darling 215 (Zhang, B. et al. 2013) and Darling 54. Reference gene was Actin. Portions of this figure are repeated from Figure 7.3a.

10.4.4 OxO enzyme activity and quantification in legacy events

Oxalate oxidase expression in Darling 4 is primarily found in vascular tissue (stem cambium and leaf veins), though there is at least some expression throughout the leaf (Figure 10.4.4a). This is expected based on the vascular-focused vspB promoter (Section 10.1), though subsequent research has revealed that other factors including sucrose and methyl jasmonate can affect expression as controlled by this promoter (Mason et al., 1993; Sadka et al., 1994).

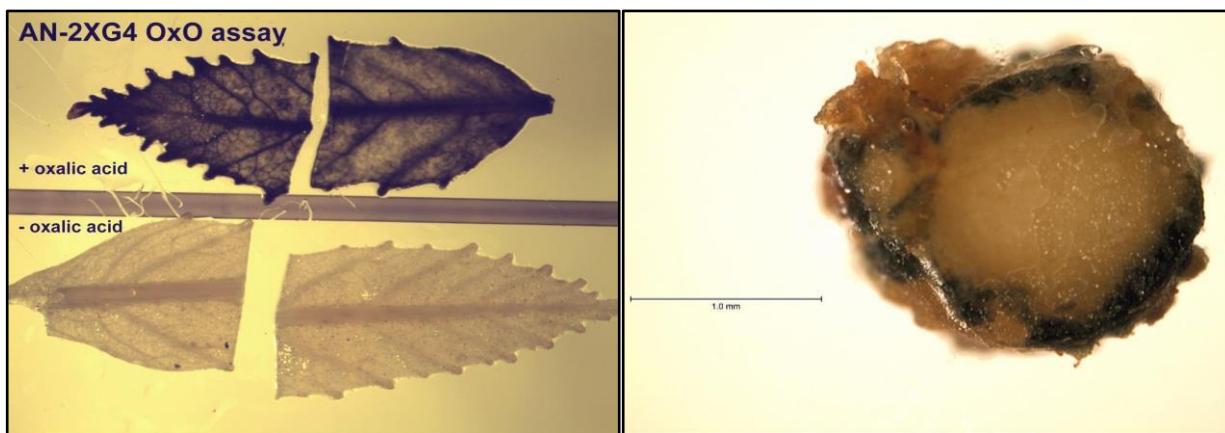


Figure 10.4.4a. Darling 4 tissues treated with the OxO histochemical assay (Section 7.4.1), showing that the heaviest expression is in vascular tissues. Young leaves at left (AN-2XG4 refers to Darling 4), small stem cross section at right (scale bar is 1 mm).

To quantify enzyme activity in various tissues, quantitative colormetric assays were performed on Darling 54, similar to those performed on Darling 58 in Section 7.4.2. Results are very similar between Darling 54 and Darling 58, as shown in Figure 10.4.4b. Pairwise t-tests were performed on each tissue type between Darling 54 and Darling 58; there were no significant differences for any tissue type.

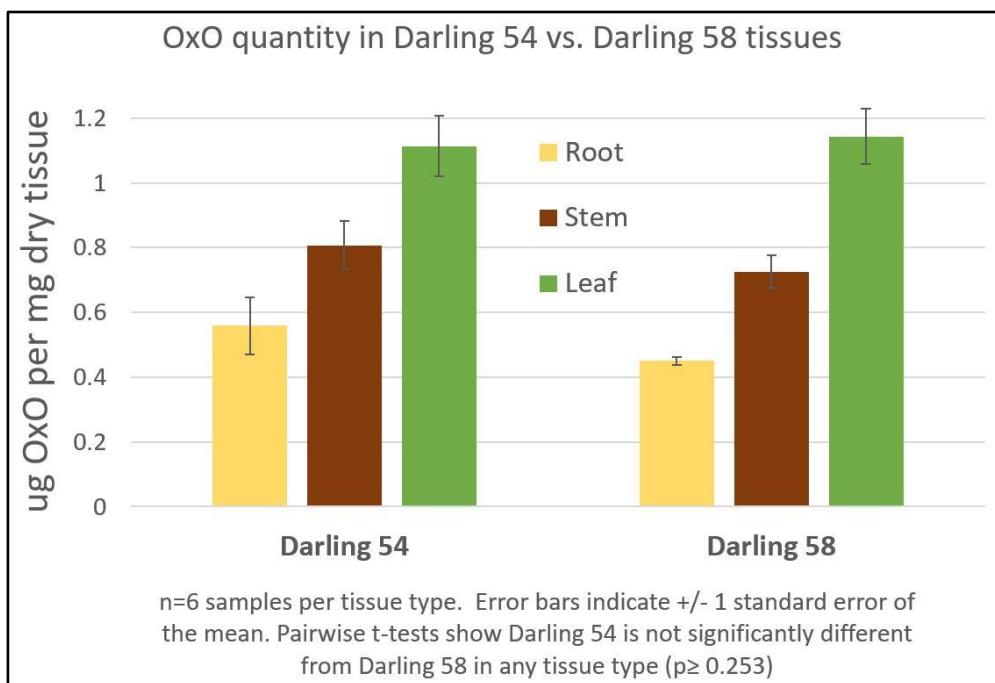


Figure 10.4.4b. Oxalate oxidase quantities in vegetative tissues of Darling 54 compared to Darling 58. (Darling 58 data are also shown in Figure 7.4.2a).

10.5 Phenotypic characterization of legacy events

10.5.1 Blight tolerance

The first replicated, controlled stem inoculation experiment on OxO-expressing American chestnut trees included the Darling 4 transgenic event, along with non-transgenic WB275-27 (American chestnut line used to produce Darling 4) and Chinese chestnut controls ($n = 3 - 5$ trees per type, repeated over two years) (Newhouse *et al.*, 2014b). These stems were a minimum of 1.5 cm diameter at ~4 cm above the ground. Wounds were 5 mm long, created with a small wire hook for consistent depth. Inoculum consisted of a 3 mm diameter agar plug with a highly virulent strain of *C. parasitica* called EP155, which was applied directly to the wound and wrapped with Parafilm for one week. Inoculations were also performed with a moderately virulent strain of *C. parasitica*; only EP155 results are shown here (Figure 10.5.1a) for consistency with other inoculation experiments in this petition. This inoculation experiment demonstrated that the OxO-expressing Darling 4 had enhanced blight tolerance compared to non-transgenic American chestnut controls, but it was not consistently as tolerant as Chinese chestnut controls.

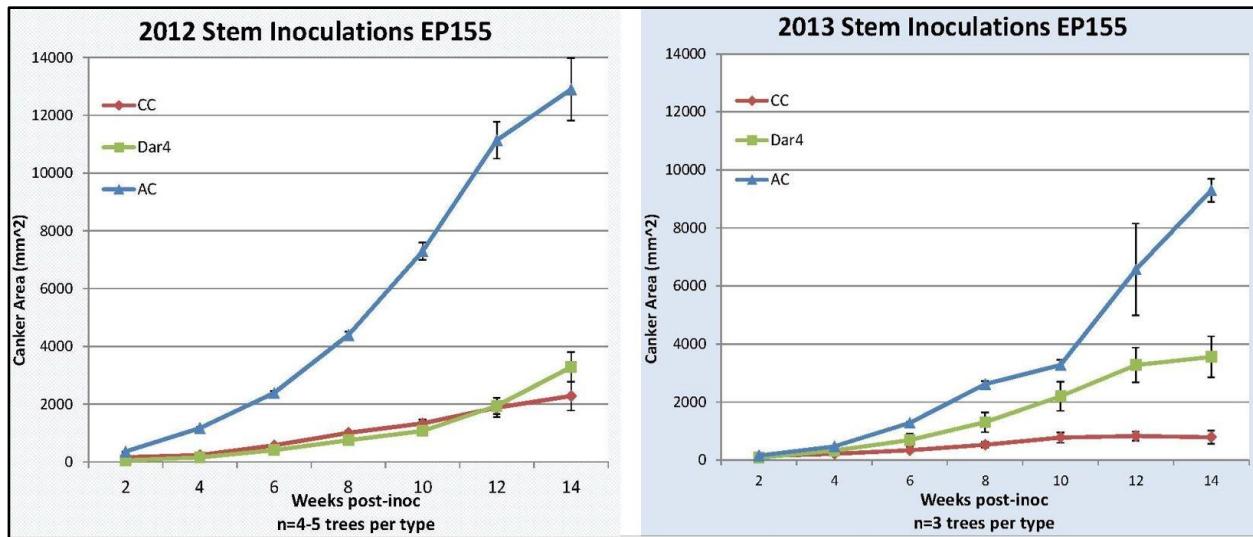


Figure 10.5.1a. Canker area progression following stem inoculations on field-grown Darling 4 (Dar4, green), with Chinese chestnut (CC, red) and non-transgenic American chestnut (AC, blue) controls. Error bars indicate +/- standard error of the mean. From Newhouse *et al.* (2014b).

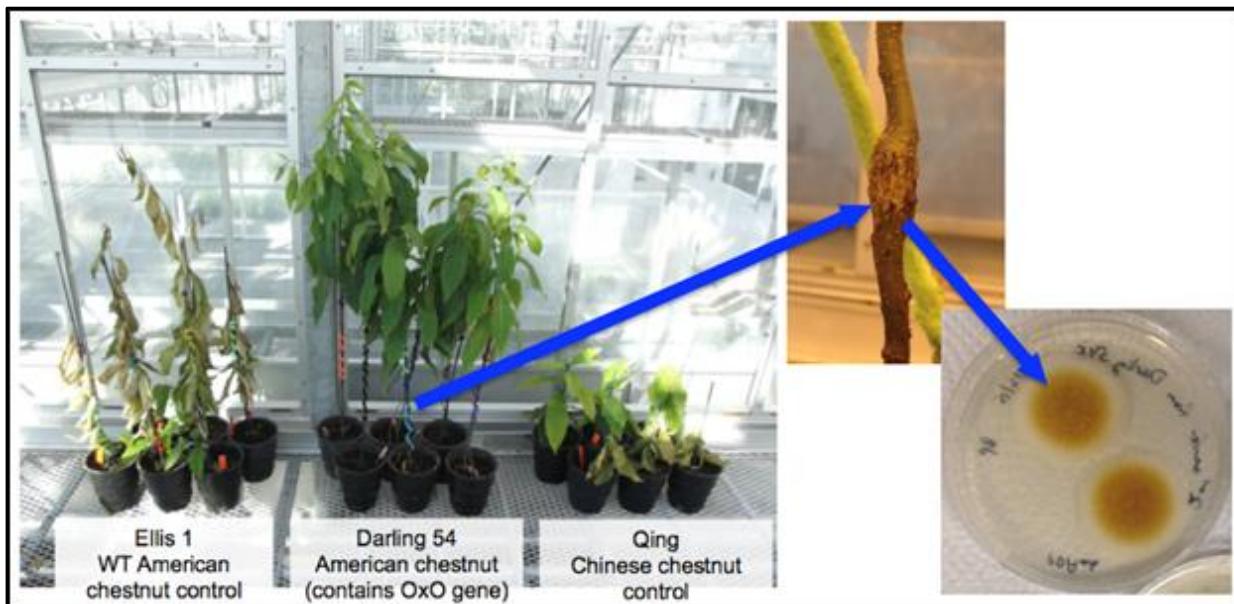
Darling 4's intermediate level of blight tolerance has been confirmed by inoculations with other strains of *C. parasitica* and leaf inoculations (both described in Newhouse *et al.*, 2014b), and natural blight infections on older trees in demonstration plots. One of the longest-lived Darling 4 trees grew in an open environment in Bronx, NY (under an APHIS permit) for several years. Transplanted to this site in 2012, this tree was later infected naturally, which resulted in several large blight cankers (two seen Figure 10.5.1b), but the tree had not wilted or died back as of spring 2018. This same tree, notably cankered but alive, was described in a recent popular-press article (Jacobsen, 2019). Figure 10.5.1b includes a close-up picture of the trunk of this Darling 4 tree, showing the fungus clearly growing and forming substantial cankers (two shown), but as shown in the figure inset, the tree thrived for several years without wilting or dying back. (It has since been coppiced to delay nut production.) While Darling 4 doesn't express as much OxO as Darling 58, it is a useful example of the tolerance mechanism in an OxO-expressing tree with documented blight tolerance (Newhouse *et al.*, 2014b).



Figure 10.5.1b. Darling 4 American chestnut in Bronx, NY, surviving despite multiple long-term blight infections. This tree was also described by Jacobsen (2019).

The first replicated stem inoculation experiment on higher-expressing 35S-OxO chestnuts included 6 plants each of Darling 54, non-transgenic American chestnut seedlings, and Qing Chinese chestnuts, inoculated in a greenhouse during the summer of 2015. Chinese chestnut controls in this experiment were shorter than American types despite their similar age, because all trees were selected to have similar stem diameters, so rate of canker girdling (i.e. fungal growth around a similar circumference) would be a meaningful comparison between types. All trees were observed daily for presence of yellowing or wilted leaves above the inoculation point, which indicates that the canker has girdled the stem to the point of disrupting the phloem (Section 3). After 6 weeks, the non-transgenic American chestnut seedlings all wilted (Figure 10.5.1c), though by that time some had re-sprouted below the inoculation point. Darling 54 transgenic American chestnuts were all healthy, but they did show swelling around the inoculation site. Three of the six Chinese chestnut controls (considered to be blight resistant under most conditions) had also

wilted above the inoculation point, indicating that this inoculation consisted of a relatively heavy pathogen load relative to the stem size. Several weeks after the inoculation, *C. parasitica* was re-isolated from stem tissue collected near the inoculation site on both Darling 54 and Chinese trees (Figure 10.5.1c inset). Identity of re-isolated fungus was confirmed by morphological comparisons with the original culture, and by subsequent re-inoculation of susceptible American chestnut tissues with the re-isolated cultures.



*Figure 10.5.1c. 2015 greenhouse stem inoculation experiment demonstrating blight tolerance of Darling 54 chestnuts compared to wild type American chestnuts (left; all wilted), and Chinese chestnut Qing (right; half wilted). At far right, re-isolation of *C. parasitica* from small stem assay cankers.*

Darling 54 was also included in the 2016 greenhouse small stem assay described in Section 8.1.2. One of the 12 Darling 54 trees in this experiment had wilted after 61 days, at which point all 12 Ellis controls had wilted. This contrasts slightly with other experiments in which none of the p35S-OxO Darling trees wilted, but still represents dramatically enhanced tolerance compared to susceptible controls. The moderate wilting on transgenic trees in this experiment may have been due in part to unrelated factors (e.g. overwatering), which led to atypical symptoms (blackened leaves) on both transgenic and non-transgenic plants.

Heritability of blight tolerance from a 35S-OxO transgenic event was first demonstrated in the summer of 2016 with stem inoculations on a pair of > 1 cm diameter T1 offspring of an event called Darling 311 (Zhang, B. et al., 2013). One of these trees had inherited the transgene and one had not. The Darling 311 event (Section 10.1) used in this demonstration includes a single copy of the same p35S-OxO transgene construct as Darling 58, and similar mRNA expression levels (not shown). Rather than direct bridging data for blight tolerance in Darling 58, however, this example is important for first confirming heritability of 35S-OxO-based blight tolerance by transgenic offspring, and for demonstrating that *C. parasitica* persists on 35S-OxO events without inflicting serious damage. Blight damage on the transgenic offspring tree is minimal and non-lethal, while

the infection on the non-transgenic full-sibling control resulted in severe damage after only a few months, consistent with blight on a susceptible American chestnut (Figure 10.5.1d).

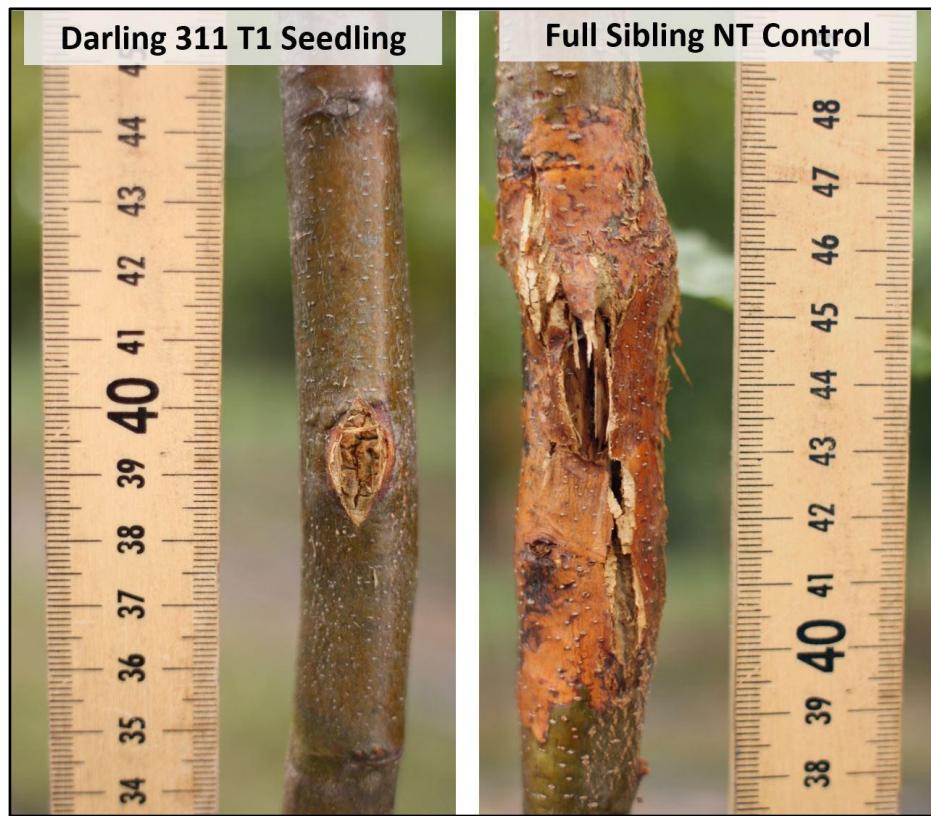


Figure 10.5.1d. Stem inoculation on Darling 311 35S-OxO T1 seedling (left) and full-sibling non-transgenic control (right) from the same cross. Photographed 13 weeks after inoculation with *C. parasitica* strain EP155.

In addition to stem inoculations, Darling 54 was used in demonstrations of oxalic acid tolerance (see also Section 6.3.1). As was later confirmed with Darling 58, transgenic Darling 54 leaf discs showed significantly less necrotic damage than Ellis non-transgenic controls after soaking overnight in 50 mM oxalic acid (Figure 10.5.1e). This experiment also included a Qing Chinese chestnut control, which showed less necrotic damage than Ellis, but more than Darling 54. This demonstrates that tolerance to oxalic acid is apparently *part of* the Chinese chestnut's defense against blight, but as described in Section 5.4, this is not due specifically to oxalate oxidase activity, and there are other mechanisms involved in Chinese chestnut blight tolerance as well.

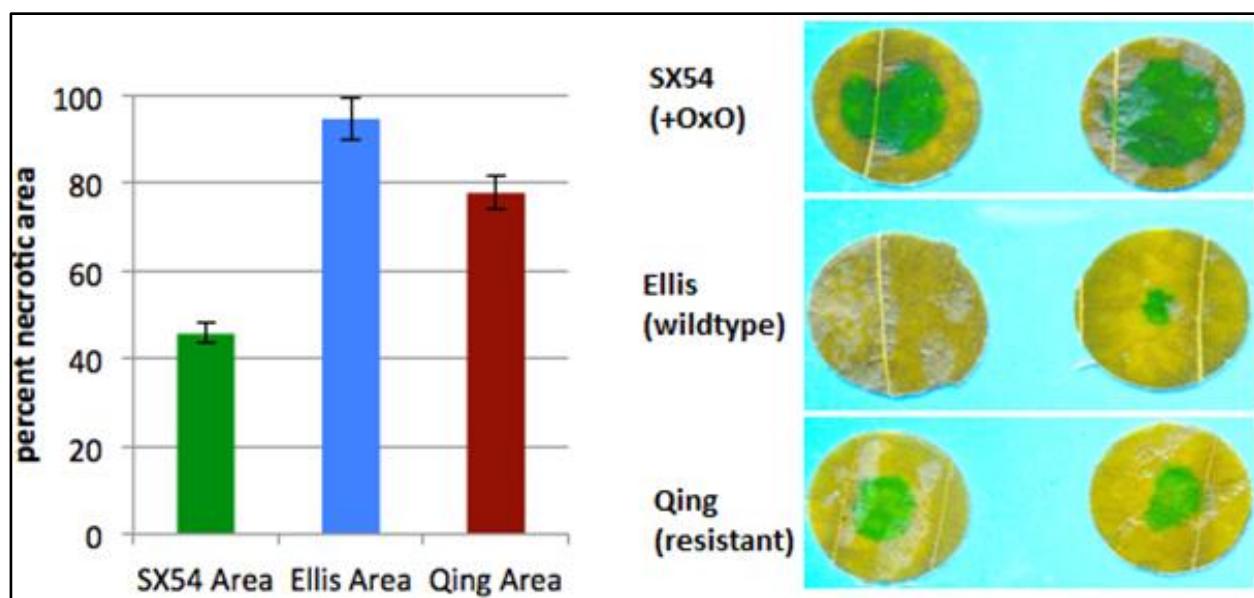


Figure 10.5.1e. Leaf disc/oxalic acid soak assay on transgenic Darling 54 (“SX54” in figure), non-transgenic American (Ellis), and Chinese chestnut (Qing), $n = 10$ discs/type. Mean percent necrosis is shown in the graph; error bars indicate \pm one standard error of the mean. At right are representative discs from each tree type.

10.5.2 Nutrition of Darling 4 T1 chestnuts

Nutritional analysis of Darling 4 T1 chestnuts was performed in the same manner as described for Darling 58 (Section 8.4.1). As seen in Table 10.5.2a, there are almost no differences between Darling 4 T1 transgenic and non-transgenic full-sibling nuts when rounded to FDA specifications for food labels. The full analysis reports with detailed (non-rounded) data are found in Appendix VII. The one difference apparent in this analysis (i.e. Vitamin C comprising 25% of the daily allowance from Darling 4 transgenic chestnuts and 15% from non-transgenic chestnuts) is still within the range seen in other non-transgenic chestnut samples (10 – 25%), and so does not demonstrate a novel change compared to traditional breeding. This confirms that transgenic expression of oxalate oxidase (even with multiple copies and additional marker genes) does not introduce novel risks to chestnuts as they would be consumed by humans or wildlife.

Table 10.5.2a. 2016 nutritional analysis results including Darling 4 T1, rounded to FDA nutrition label specifications. Non-transgenic controls are also shown in Table 8.4.1a; they are repeated here for comparison with Darling 4 T1 nuts analyzed at the same time. See Section 8.4.1 and Appendix VII for more details.

| Chestnut nutrition and composition information (2016 analysis only, with Darling 4) | | | | | | | |
|---|-------------------------------------|------------------------------------|---------------------------------|---------------------------------|----------------------------|--------------------|-------------------|
| Chestnut type: | Darling 4 T1 Transgenic American | NT Full Sibling Of Darling 4 T1 | NT American (Wisconsin) x OP | NT American (Moss Lake) x OP | NT American (Zoar) x OP | NT American x B3F3 | European Chestnut |
| Serving Size | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~15 nuts) | 50g (~5 nuts) |
| Calories | 120 | 120 | 120 | 80 | 110 | 120 | 90 |
| Cal from fat | 40 | 40 | 30 | 10 | 20 | 40 | 10 |
| Total fat (g) | 4.5 | 4.5 | 3.5 | 1.5 | 2.0 | 5.0 | 0.5 |
| Sat Fat (g) | 0.5 | 0.5 | 0.5 | 0 | 0.5 | 0.5 | 0 |
| Trans Fat (g) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Polyunsat fat (g) | 1 | 1 | 0.5 | 0.5 | 0.5 | 1 | 0.5 |
| Monounsat fat (g) | 3 | 3 | 2 | 0.5 | 1.5 | 3 | 0 |
| Sodium (mg) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total Carbohydrate (g) | 19 | 19 | 19 | 17 | 20 | 18 | 21 |
| Fiber (g) | 5 | 5 | 5 | 3 | 5 | 5 | 4 |
| Protein (g) | 1.5 | 1.5 | 2.0 | 1.5 | 2.5 | 1.5 | 1.0 |
| Vit C % (RDI 60mg) | 25 | 15 | 15 | 20 | 15 | 10 | 25 |
| Ca % (RDI 1000 mg) | 1 | 1 | 3 | 2 | 3 | 1 | 1 |
| Fe % (RDI 18mg) | 4 | 4 | 4 | 3 | 4 | 4 | 6 |

10.6 Environmental interactions with legacy events

10.6.1 Mycorrhizal colonization of greenhouse-grown Darling 4 roots

As described previously (Section 9.1.1), mycorrhizae are soil fungi that form ecologically important relationships with plants, disruption of which could potentially indicate plant pest risks. This section describes experiments performed with Darling 4 (Sections 10.1 and 10.2) as bridging data for the Darling 58 event, and adds valuable context with extraspecific plant controls and field conditions beyond mycorrhizal experiments conducted with Darling 58 alone (Section 9.1.1).

D'Amico *et al.* (2015) compared ectomycorrhizal fungal colonization on the Darling 4 transgenic American chestnut to wild-type American chestnut and other Fagaceae species. A greenhouse bioassay used mycorrhizal inoculum (field soil) collected from two sites ("upper shelterwood" and "lower shelterwood") with different soil types and land use histories. Justification for the use of field soil is provided by Dulmer *et al.* (2014), who found that soils collected from stands once populated by American chestnut, but currently dominated by northern red oak, work well as mycorrhizal inoculum for chestnut. The soil was collected from 30 random points at each of the two sites on ESF property. One liter of soil was gathered from each point and pooled together to amass 30 liters per site. The soil was mixed with sand and potting mix (Fafard Super-Fine Germinating Mix; Sun Gro Horticulture, Agawam, MA, USA) in a 1:1:2 (soil:sand:potting mix) ratio, using a rotating-drum compost bin. Six seeds each of American beech (AB), northern red oak (RO), Qing Chinese chestnut (CC), wild-type American chestnut (seed lot "Zoar" from western NY) (WA),

and (Chinese x American) x American backcross hybrid (HY) as well as six plantlets of Darling 4 transgenic American chestnut (TA) were potted in containers with the soil mixture from one site while six seeds/plantlets of each tree type were potted in containers with the soil mixture from the second site. All plants were placed in a greenhouse in a completely randomized design.

After approximately 1 year of growth in the greenhouse, root tips were harvested from each of the surviving trees ($n = 67$). The number of colonized root tips (approximately 150 – 200 per plant) was counted under a dissecting microscope and fungal species were identified using both morphological and molecular techniques. Molecular techniques consisted of DNA extractions from fungal root tips using a CTAB protocol outlined by Gardes and Bruns (1993). PCR was performed using primers for an internal transcribed spacer (ITS) region, and positive products were digested separately with three restriction enzymes: Hinfl, Alul, and DpnII (New England Biolabs, Inc., Ipswich, MA, USA). Products with matching band patterns for all three restriction enzymes were classified as one digest group or restriction fragment length polymorphism (RFLP) type, and DNA from products of each RFLP type was sequenced. Each sequence was searched in the NCBI BLAST GenBank database¹⁰. Matches of 97% or greater were considered species-level matches, between 95 and 96% were considered genus-level matches, 90 and 94% were family-level matches, and less than 90% were order-level matches. Analysis of variance (ANOVA) was used to compare total percent colonization, species richness, and species abundance between tree type and soil type, and a Waller-Duncan test was used to compare pairs of means. All analyses were performed in SAS (version 9; SAS Institute Inc., Cary, NC, USA).

Total ectomycorrhizal colonization (Figure 10.6.1a) varied more by soil type than by tree species. Individual fungal species varied in their colonization rates, but there were no significant differences between mycorrhizal colonization on transgenic and wild-type chestnut roots. Further analyses of fungal species richness (Figure 10.6.1b) and colonization by individual fungal groups (Figure 10.6.1c) also indicate that there are no differences in mycorrhizal colonization between transgenic and wild-type American chestnut. The findings confirm that the transgenic Darling 4 American chestnut does not significantly differ from the wild-type American chestnut in colonization by ectomycorrhizal fungi and therefore, presence of the OxO transgene does not present novel plant pest risks that might interfere with these relationships.

10 Available at: <http://www.ncbi.nlm.nih.gov/GenBank/>

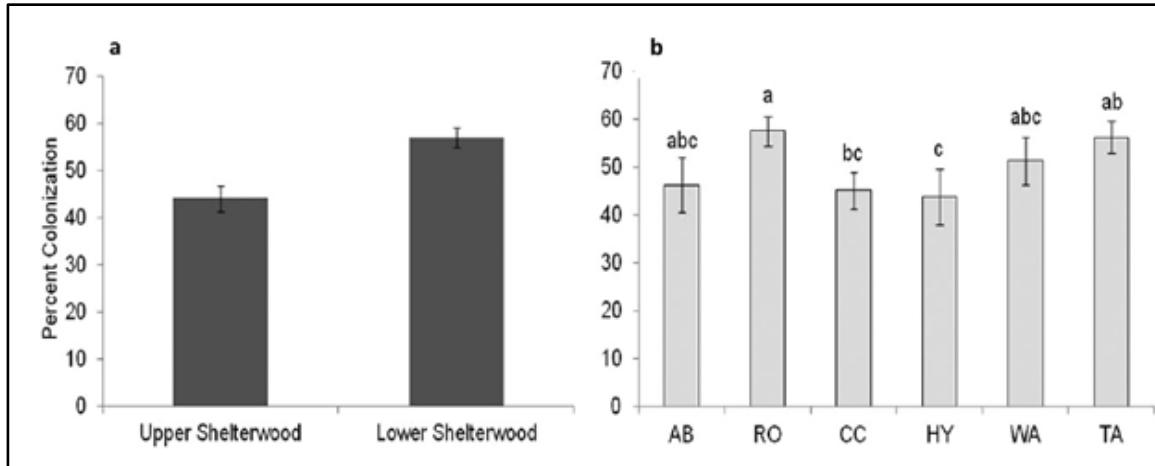


Figure 10.6.1a. Percent colonization (number of EM-colonized root tips counted/total number of root tips counted) by (a) soil site ($P < 0.0003$) and (b) tree species. Means sharing a common letter are not statistically different based on the Waller-Duncan pairwise comparison test with an experiment-wise error rate of 0.10); bars represent ± 1 SE. See text for tree type abbreviations.

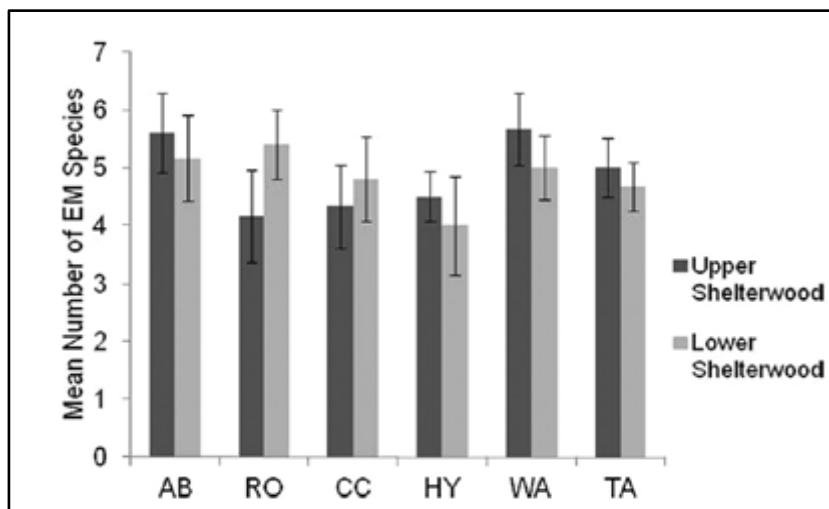


Figure 10.6.1b. Ectomycorrhizal (EM) fungal species richness by tree type and soil site. Column height indicates the number of EM fungal species present per tree type. The effect of tree species was not statistically significant ($P = 0.49$), so Waller-Duncan pairwise comparison tests were not conducted. Bars represent ± 1 SE. See text for tree type abbreviations.

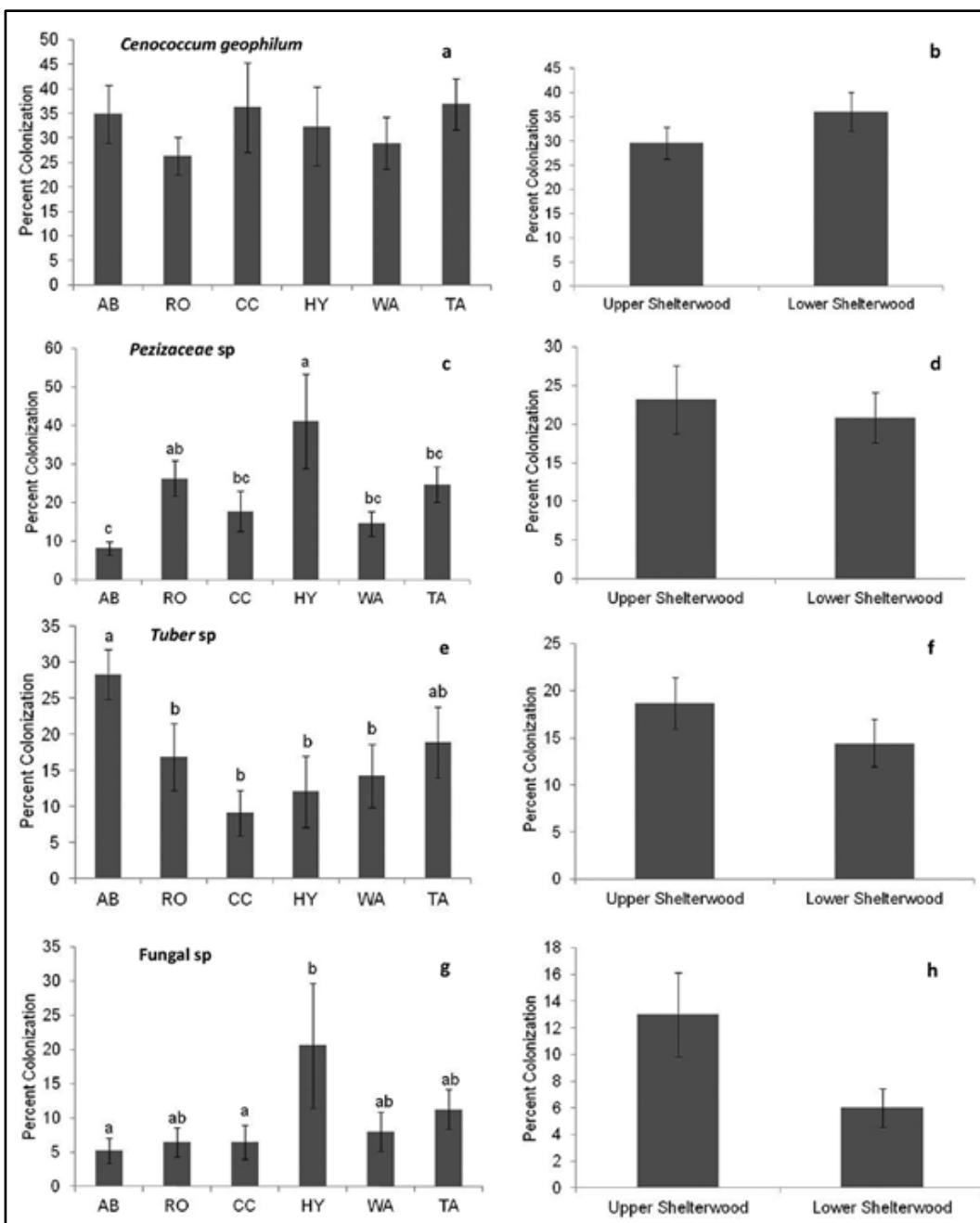


Figure 10.6.1c. Percent mycorrhizal colonization by tree type and soil site. Colonization rates based on tip counts of *Cenococcum geophilum* (a and b), *Pezizaceae* species (c and d), *Tuber* species (e and f), and other fungal species (g and h) comparing tree species (see text for abbreviations) and soil types. Species means sharing a common letter are not statistically different based on the Waller-Duncan pairwise comparison test (experiment-wise error rate of 0.10), and bars indicate ± 1 SE.

10.6.2 Mycorrhizal colonization of field-grown Darling 4 roots

Besides evaluations on greenhouse-grown Darling 4 roots, mycorrhizal colonization was also observed on transgenic (Darling 4 and Darling 5) and wild-type American chestnuts planted outdoors in permitted field sites. Tourtellot (2013) compared ectomycorrhizal fungal colonization

on transgenic American chestnuts to wild-type American chestnuts, Chinese chestnuts, and hybrid chestnuts in four separate plots near Syracuse NY, which included two open field plantings and two shelterwood plantings. The study included six American chestnut transgenic events produced from tissue culture: Darling 4, Darling 5, Wirsig, Hinchee 1, Hinchee 2, and AN-2G3; one non-transgenic line from tissue culture: WB-275-27; two non-transgenic American chestnut seedlings: Lasdon and Zoar; two Chinese chestnut seedlings: Cropper and Qing; two first generation backcross (Chinese x American) x American chestnut seedlings: K-L BC1 and GR68-B1; and one complex hybrid seedling of Japanese, Chinese and American chestnut: Luval's Monster. Only the Darling 4 and Darling 5 events are being used comparisons to Darling 58 (Section 10.2), since they share the same OxO transgene. Hinchee, Wirsig, and AN-2G3 events are not intended for direct comparisons to Darling 58 due to additional transgenes or construct differences. Each of the four planting sites consisted of 70 trees planted on two by three meter spacing. Rows were divided into five replicated blocks, each block consisting of two rows. Each chestnut line was represented once in each block, and were positioned randomly within blocks. Due to availability, trees were planted over two years, with dead plants replaced in the spring or fall after initial planting. Tissue cultured plantlets had a higher mortality than seedlings.

Root samples were dug up two years after planting, tracing each root back to the source plant to confirm identity. Both morphological and molecular identification were similar to methods used in the greenhouse mycorrhizae study by D'Amico *et al.* (2015). Samples were examined under a dissecting microscope at 50x magnification and ectomycorrhizal fungal colonization rates were quantified as the number of ectomycorrhizae divided by the total number of root tips. Ectomycorrhizae were categorized into morphotypes based on color, branching pattern, the characteristics of extramatrical hyphae, the presence of cystidia, and the texture of the fungal mantle (Figure 9.1.1a). Typically 8 to 12 morphotypes were found per 12.5 cm sample. For molecular analysis, DNA was extracted from each morphotype, RFLP analysis was performed on PCR products from the ITS region, digest banding patterns were compared, and representative samples of each RFLP type were sequenced to confirm identity via GenBank.

Analysis of variance (ANOVA) was performed using SAS software version 9.2 for Windows, copyright 2002 – 2008 by SAS Institute Inc., Cary, NC, USA. Plant type treatments were compared using non-parametric multivariate analysis of variance (Anderson 2001, McArdle and Anderson 2001), also known as PERMANOVA, using DISTLM v.5 (Anderson 2004) when single replicates were available.

Ectomycorrhizal fungal colonization rates increased with the length of time the plants were in the ground. Because the chestnuts were planted or replaced at different times, analyses were separated based on how long plants had been in the ground: 4 months, 12 months, 18 months, 24 months, and 28 months. Colonization rates between these time classes were significantly different (Tourtellot, 2013), and therefore colonization rates were only compared within the time classes.

As with the greenhouse study, there were no significant differences in colonization of Darling 4 chestnut roots by mycorrhizal fungi compared to non-transgenic WB275-27 or other American chestnut controls (Figure 10.6.2a). This experiment showed similar results for Darling 5 as well, which was not included in the greenhouse study. Ectomycorrhizal fungal colonization was

generally lower for all tree types in open field sites than in shelterwood sites, and colonization rates on all tree types generally increased with time in the ground. ANOVA analysis of all transgenic and non-transgenic tree types found significant differences only with the Hinchee transgenic events that had been in the ground for 4 months, and these differences disappeared after one growing season. There were no significant differences between OxO events (Darling 4 or Darling 5) and any American chestnut controls (WB275 isogenic line or unrelated American seedlings). Most root samples in a given time class, regardless of tree type or transgene status, showed similar ectomycorrhizal colonization, and there was no indication that tree type or transgene status differentially affected colonization by any species or genera of ectomycorrhizal fungi. Figure 10.6.2a shows excerpts of colonization data from Tourtellot (2013) in order to focus on OxO events relevant to bridging. Note that in this excerpt figure, time classes for which $n < 2$ for Darling 4 or non-transgenic American controls, and data for other plant types (e.g. Hinchee, Wirsig, and other *Castanea* species/hybrids), are not shown.



Figure 10.6.2a. Mycorrhizal colonization of field-grown chestnut roots; summary chart above and detailed results below. All data are excerpts from Table 4 in Tourtellot (2013). In that original publication, WB275-27 is called C-0, Darling 5 = Ox-1a, Darling 4 = OX-2; Lasdon is called "Am-A", and Zoar is called "Am-B", names have been corrected here for clarity. Not all plant types were present in all age classes (time in ground) due to mortality/re-planting schedules. Error bars indicate +/- one standard error of the mean.

10.6.3 Darling 4 leaf litter: decomposition, Carbon:Nitrogen ratios, and fungal diversity

Gray and Briggs (2015; see also Gray, 2015), performed a study to determine whether leaf litter decomposition rates varied between transgenic American chestnut and non-transgenic chestnut controls. In addition, concentrations of carbon (C), nitrogen (N), C/N ratio, and micronutrient components were analyzed over a 30-month period, and fungal diversity was assessed in leaf litter. These experiments were performed at a 100-year-old oak-hickory shelterwood plot near Syracuse, NY. The goal was to determine whether ecological function of transgenic trees was equivalent to that of non-transgenic American chestnuts or traditionally bred hybrids. Leaf litter decomposition plays a crucial role in maintaining site fertility and productivity in forest ecosystems (Prescott, 2005). A biologically significant change in leaf litter quality during decomposition could affect the general successional pattern of fungal functional groups such as litter-decomposing fungi, wood-decomposing fungi, and mycorrhizal fungi. Therefore, ecological changes including plant pest risks could conceivably result from substantial changes to decomposition rates, nutrient components, or fungal diversity in leaf litter compared to wild-type trees.

Four types of leaf litter were collected and used in the leaf decomposition study: a pooled hybrid sample consisting of two (Chinese x American) x American chestnut hybrids (GR68-B1 and K-L-BC1); Darling 4 transgenic American chestnut; Hinchee 1 transgenic American chestnut (Section 10.1); and a wild-type American chestnut seedling line called Zoar. Note that the Hinchee event contains additional transgenes not found in Darling 58 (see Table 10.1a), and therefore is not intended for direct comparisons or bridging data with Darling 58. Ten grams of dried leaf tissue from each tree type was put into 2 mm mesh litterbags and placed directly on the forest floor. A total of 120 litterbags were deployed in the field at 30 different points, lining the perimeter of the plot. At each point, a set of four litterbags (each containing one leaf type) were placed on top of the soil in order to simulate actual leaf litter occurrence and account for the effects of microclimate and slope.

Mass loss occurred more quickly than anticipated based on previous experience with red maple (Briggs unpublished); all leaf types had lost more than 80% of their mass after 12 months. Mass remaining did not differ among litter types after 12, 18, 24, and 30 months of field exposure ($p > 0.05$, Figure 10.6.3a). There was no interaction between litter type and time for mass remaining ($p = 0.40$).

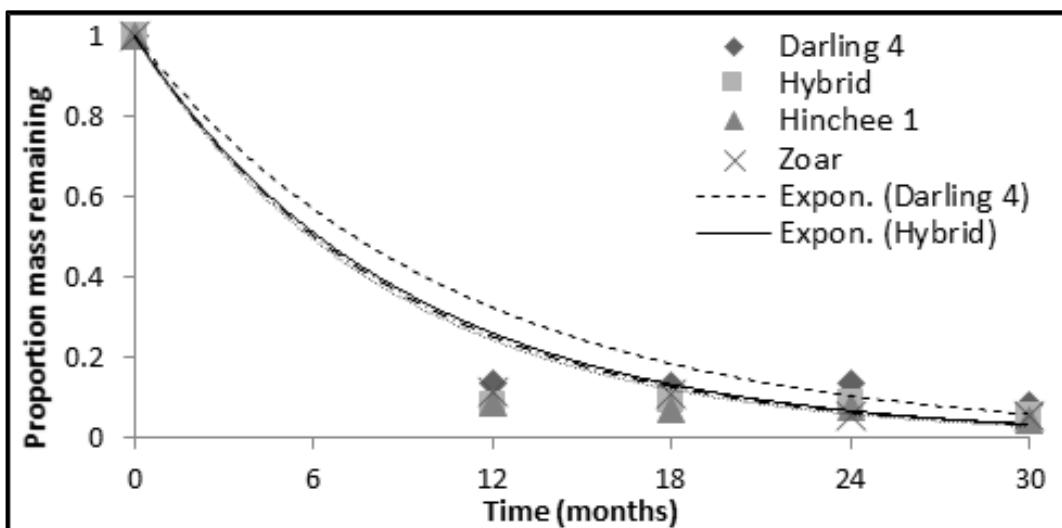


Figure 10.6.3a. Chestnut leaf decomposition over a 2.5-year period. Nonlinear model ($Y = e^{-kt}$) fit to proportion of ash-free mass remaining in decomposing transgenic (Darling 4, Hinchee 1), Hybrid and Zoar leaf litter.

The results of this study do not show significant variation in the rate of litter mass loss between Darling 4 transgenic American chestnuts, Zoar non-transgenic American chestnut, and hybrid chestnuts over the 2.5 year in situ incubation period, at least during late stage decomposition. This is consistent with other studies reporting minimal variation between decomposition of transgenic and non-transgenic plant tissues (Tilston *et al.*, 2004; Seppänen *et al.*, 2007). While transgene presence does not appear to have a significant effect on chestnut leaf litter degradation, other factors like disease presence have been shown to affect degradation of chestnut leaves (Pazianoto *et al.*, 2019), so this is an important factor to consider when evaluating environmental risks of potential conservation strategies.

Throughout the 2.5-year decomposition period in this study, leaf C/N ratios varied slightly among litter types (Figure 10.6.3b), with Zoar having the highest initial C/N ratio and Hinchee 1 having the lowest C/N ratio ($p < 0.001$), and all ratios decreased over time ($p < 0.001$). There was no interaction between litter type and time for C/N ratio ($p = 0.45$) (Figure 10.6.3b). Of the seven other elements analyzed (Ca, P, K, Mg, Mn, Na, and Al), only Ca and P concentrations differed among litter types throughout the 2.5-year field trial, with Ca concentration increasing and then decreasing after two years in all litter types except for Hinchee 1. As mentioned above, Hinchee 1 (the only type with significant differences), has an additional gene of interest (an antimicrobial peptide gene) beyond OxO, as well as having multiple insert copies, so it is not necessarily directly comparable to Darling 4 or Darling 58 (see also Section 10.1). These studies suggest that the insertion and expression of the OxO transgene in Darling 4 American chestnut trees does not have any measurable effect on the mineralization of elements examined during decomposition.

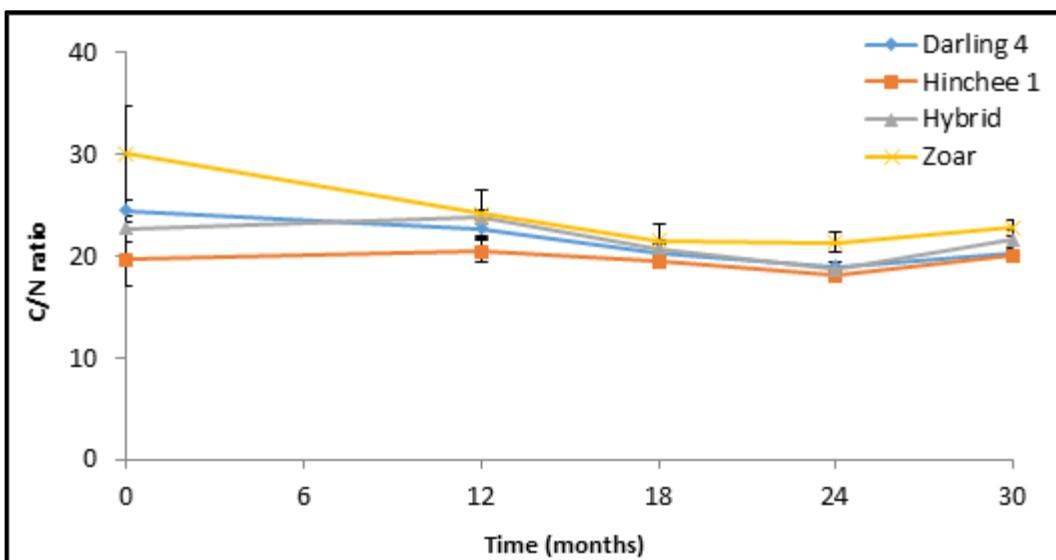


Figure 10.6.3b. Mean C/N ratios for transgenic (Darling 4, Hinchee 1), Hybrid and Zoar leaf litter over a 2.5-year decomposition period. $n = 2$ at initial time point; $n = 6$ at 12, 18 and 24 months; $n = 12$ at 30 months. Error bars represent ± 1 standard error of the mean.

In addition to the decomposition study above, Gray (2015) investigated the effect of transgenic American chestnut leaf litter on the diversity of fungal species and functional groups colonizing litterbags during a five-month field trial near Syracuse, NY. Fungal hyphae were harvested from litter bags to identify the fungi using PCR-based methods (Gardes and Bruns, 1993). This study aimed to discover more information about the fungal species that decompose American chestnut leaf litter and to determine if transgenic chestnut litter is ecologically equivalent to wild-type in terms of diversity of fungal colonizers via diversity indices and species richness. This study used three of the same leaf types (Zoar, Darling 4, and Hinchee 1) and the same forest location as the previous decomposition study.

Hyphae from each litterbag were initially sorted by morphotype, and then characterized using restriction fragment length polymorphism (RFLP) followed by sequencing one candidate of each RFLP type. Species-level matching was determined by identical RFLP matches with digests of two enzymes, *HinfI*, and *DpnII*. Operational taxonomic unit (OTU) names were assigned based on top NCBI matches to determine species, genus, or family level identity depending on available data and similarity of sequences in the database. Species diversity values for each litter type were computed in Estimate S (Colwell and Elsensohn, 2014) using estimated species richness, Shannon, and inverse Simpson indices. Absolute frequency is defined as the proportion of bags in which the OTU was observed. Gray (2015) used RFLP type diversity as a proxy for species diversity. Species richness was determined for each litter type by averaging the number of fungal types found in each individual litter bag. Once the successful morphological types had been sorted into RFLP types and sequenced to OTUs, Estimate S was used to estimate the species richness of the population after 100 randomized runs.

This interpolation found that Darling 4 litter species richness was not different from Zoar or Hinchee 1. Fungal species diversity differed significantly between leaf types using both the Shannon index ($p < 0.001$) and the inverse Simpson index ($p < 0.001$), with Zoar having the highest

species diversity, followed by Darling 4 and then Hinchee 1 (Table 10.6.3a). As described above, differences in the Hinchee 1 event are not necessarily applicable to Darling 58, so data from Darling 4 are of more relevance here.

Table 10.6.3a. Mean species richness, Shannon diversity index and Inverse Simpson index of Zoar, Darling 4, and Hinchee 1 leaf litter communities after interpolation using 100 replications with Estimate S. Numbers in parentheses represent the standard error of the mean. From Gray (2015).

| | S (est) | Shannon | Inv Simpson |
|-----------|-----------|-----------|-------------|
| Zoar | 4.0 (1.1) | 1.3 (0.3) | 3.7 (0.8) |
| Darling 4 | 3.0 (1.0) | 1.0 (0.3) | 2.8 (0.8) |
| Hinchee | 2.0 (0.6) | 0.7 (0.3) | 1.9 (0.4) |

Of the 73 morphological types originally found within the 30 litterbags used in this study, 39 were successfully run through PCR and RFLP analyses. The 39 morphological samples were sorted into 19 distinct RFLPs. These RFLPs were grouped into 15 distinct OTUs using a cutoff of 97% sequence similarity. The fungal OTUs were categorized into either ectomycorrhizal (EM) or saprotrophic groups. EM groups were found to dominate all litter types after five months of decomposition. With all 10 samples of each litter type combined, Zoar leaf litter had 84.6% EM fungal samples and 15.4% saprotrophic samples. Darling 4 litterbags were found to have 72.7% EM fungal samples and 27.3% saprotrophic samples. Hinchee 1 litterbags had 70.0% EM fungal samples and 30.0% saprotrophic fungal samples. Jaccard indices were calculated in Excel for each pair of communities. The Zoar community was found to be equally similar to both Darling 4 and Hinchee 1 communities in the number of OTUs shared with a Jaccard index of 0.33 for both sets of communities.

The analysis found fungal species diversity to be higher in Zoar than in Darling 4. Diversity in Hinchee 1 leaf litter was lower than Zoar and Darling 4 (Table 10.6.3a). The largest difference, that between Hinchee 1 and Zoar, may be caused by the antimicrobial peptide gene present in the Hinchee events (See Section 10.1; because of this, Hinchee 1 is not intended to be used for bridging data). Alternatively, this difference, and the smaller difference between Zoar and Darling 4, may be an artifact of low sample size. Only 10 litterbags were deployed for each leaf type, and rarefaction curves showed the number of OTUs continuing to increase for all leaf types, indicating that the study did not fully capture the diversity of fungal OTUs that would ultimately colonize leaf litter of the three leaf types. Because of this, Gray warns that despite statistical significance, inferences about differences in diversity are "most likely premature," and that "overall, the process of genetic engineering using the transgene oxalate oxidase does not appear to have any measurable effect on the diversity of fungi that colonize leaf litter."

10.6.4 Persistence of OxO activity in Darling 4 leaves

In order to test persistence of the OxO enzyme in senescent leaf tissue, OxO activity was tested with a histochemical assay (Section 7.4.1) in leaves from transgenic Darling 4 T1 trees. Briefly, this assay involves two tissue pieces collected from a single leaf, which are placed into histochemical assay solutions (Dumas *et al.*, 1995). Leaf pieces containing active OxO enzyme show a dark blue/black color in the tube with oxalic acid substrate, but no color change is observed where OxO is inactive or not present. Portions of this experiment have since been repeated with Darling 58 leaves (Section 9.4).

In the late fall of 2013, when leaves were starting to drop, several leaves were removed from a Darling 4 transgenic T1 tree and three conditions were tested: storage in a mesh bag on the ground outside, storage in a refrigerator at 4°C, and storage in a freezer at -20°C. Leaf pieces were regularly collected from all storage conditions for histochemical testing of oxalate oxidase activity. Initial tests on green leaves attached to the tree showed a black ring in the test solution with oxalic acid, confirming that the assay was working and these leaves had OxO activity. Leaves left in mesh bags outdoors continued to show OxO activity for approximately one week after being removed from the tree, but as soon as the leaf dried and started to turn brown, OxO activity was no longer detectable. Leaves stored in a plastic bag at 4°C retained activity longer, with detectable activity up to approximately one month after removal from the tree. Leaves stored in a plastic bag at -20°C showed activity for at least one year after removal from the tree, suggesting that OxO activity can be preserved under artificial conditions. According to the field-realistic condition of leaves falling from a tree, OxO activity was found to cease after approximately one week.

10.6.5 Native plant responses: natural colonization near transgenic chestnuts

A preliminary study was performed on field-grown transgenic American chestnuts in permitted field plots to determine if colonization of different plant species was affected by the presence of nearby transgenic chestnut trees. Three sites near Syracuse, NY were planted with transgenic ($n = 30/\text{plot}$) and non-transgenic ($n = 40/\text{plot}$) chestnut trees: one open field plot, and two shelterwood plots. Another open field plot near Tully, NY was used as well for a total of four plots. Transgenic American chestnut lines in this study included Darling 4, Darling 5, Hinchee 1, Hinchee 2, Wirsig, and AN-2G3 ($n = 5$ of each type per plot, see Section 10.1 for descriptions of all events), all produced from tissue culture. (Only Darling 4 and Darling 5 are intended for use in bridging data to Darling 58 from this study; other events have additional genes that are not directly relevant to Darling 58.) The non-transgenic isogenic line for the transgenic events, WB275-27, was the only non-transgenic control produced from tissue culture. The rest of the non-transgenic controls were seedlings from the following seed lots: two pure American chestnuts (Zoar and Lasdon), two Chinese chestnuts (Cropper and Qing), two first-generation backcross hybrids, and a complex hybrid called Luvall's Monster.

After each chestnut tree was planted, a 3' x 3' permeable mat was laid down around its base, to suppress competing plants that may have colonized the location. The following spring, when the chestnut trees had time to grow and adapt to the location, the mats around the trees were removed, leaving a 1' x 1' mat in place around the stem to prevent direct competition with the growing trees. Two months later, the sections previously containing the mats were checked for plant colonization (colonization defined as a plant having either a stem or node rooted within the

area of the 3' x 3' mat). Out of 280 subplots (i.e. observed areas around a single tree), less than 1% had any plants growing. Trailing plants like *Rubus* spp. (wild raspberry and blackberry species) and *Parthenocissus quinquefolia* (Virginia creeper) that were sprawling over the area were not counted as colonizers since they were not rooted in the actual plots. Because of the low colonization rate, the chestnut trees were left for an additional year before data were collected. This allowed any species in the seed bank to germinate and gave adequate time for the plants outside of where the mats were located to move in and colonize. This was a blind study, as the data collector did not know what trees were transgenic until after the analysis was complete.

For each subplot, a 1 m² quadrat was used to identify plant species and to determine percent cover. Six cover classes were used: class 1 = < 5%, 2 = 5 – 25%, 3 = 25 – 50%, 4 = 50 – 75%, 5 = 75 – 95%, 6 = > 95%. Twenty-five species were identified in the Syracuse open area, twenty-six species were identified in the Tully open area, twenty-six species were identified in the first Syracuse shelterwood area, and fourteen species were identified in the second Syracuse shelterwood area. Some, but not all of the species were found in more than one location. Only *Fragaria* (strawberry species) was seen in all four plots. Each plot was fully sampled within one week of starting the count, to make sure seasonal changes did not affect plant community composition. A two-sample T-test was used to compare the abundances of each plant species in transgenic and non-transgenic subplots.

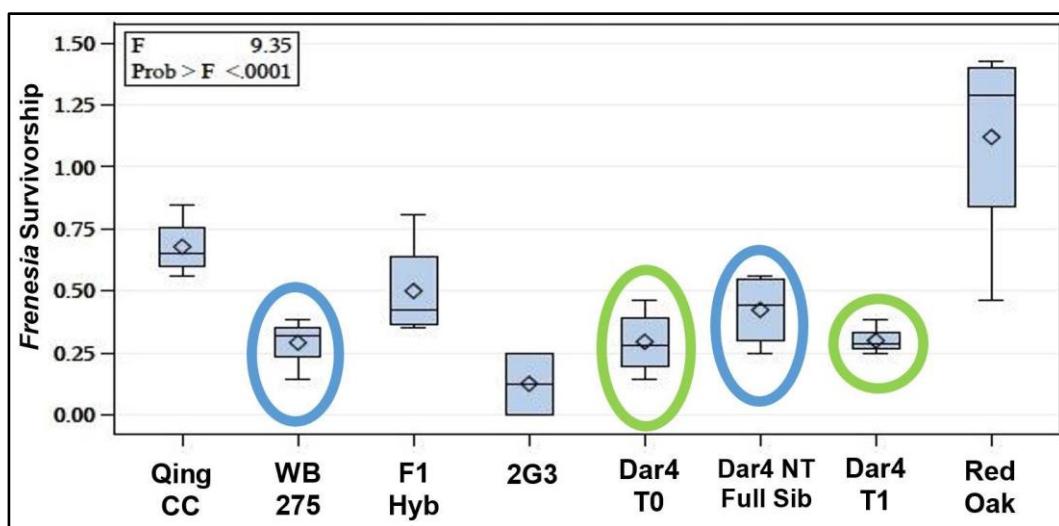
There was no significant difference between the colonizing plant communities ($p > 0.05$) found next to the transgenic trees and those found next to the non-transgenic trees in the three plots near Syracuse NY, according to either a two-sample T-test or a Mann-Whitney test. Therefore, it can be concluded that these transgenic American chestnut trees did not affect the germination or growth of other nearby plant species. High mortality (63%) of tissue culture-generated transgenic trees and 100% mortality of non-transgenic tissue culture controls in the Tully plot prevented meaningful analysis from this location. Differences in plant colonization and community diversity at the Tully plot could be explained by uneven ages of tissue culture and seed-origin trees, and disturbance resulting from re-planting tissue culture trees. These confounding factors highlight the need in future studies to compare trees produced from similar methods, i.e. to use only tissue culture-generated non-transgenic controls when testing tissue culture-generated transgenic trees, or to use exclusively seedling offspring.

10.6.6 Aquatic insect herbivory on chestnut leaves

A study was conducted on Caddisfly (*Frenesia difficilis*), comparing survival of larvae reared on leaves of various tree species including transgenic and non-transgenic American chestnut (Sweeney *et al.*, in prep). Caddisfly larvae were selected for this study because they belong to a feeding guild known as leaf shredders (Wallace and Anderson, 1996), which serve an ecologically important role of both consuming and breaking down deciduous leaves, which can enter streams in copious quantities and form bases for aquatic ecosystems (Tiegs *et al.*, 2008). Shredder species such as caddisflies are known to respond significantly to chemical or trophic characteristics that vary between leaf types (Sweeney, 1993), and they are specifically known to be sensitive to environmental variations and pollution (Hilsenhoff, 1987). Finally, *F. difficilis* is widely distributed in northeastern North America (Cummins *et al.*, 1996), overlapping considerably with the original range of the American chestnut tree.

Caddisfly egg masses were allowed to hatch in the laboratory, and larvae were transferred to experimental chambers containing 15°C aerated water and sieved stones (used by the insects to form protective cases). Fifty larvae were added to each chamber (4 replicates for each of 8 leaf types), and an additional 30 larvae were added to each chamber after 7 days. Leaves were conditioned by soaking for eight days in 0.45 µm filtered spring water in individual glass jars kept at 20°C without light, and added to experimental chambers as needed to keep food levels non-limiting. Eight leaf types were used in the experiment: transgenic Darling 4 and Darling 4 T1 offspring inheriting the transgene, non-transgenic WB275-27 (isogenic to Darling 4) and Darling 4 T1 offspring not inheriting the transgene, transgenic event AN-2G3 (containing only the marker gene pGFP), F1 (Chinese x American) chestnut, Chinese chestnut, and northern red oak. After 70 days, caddisfly survival was recorded for each chamber.

According to ANOVA followed by Tukey's HSD, survival of caddisfly was significantly lower on all American chestnut types compared to Chinese chestnut and northern red oak ($p < 0.05$), but no significant differences were found in any pairwise comparison among American chestnut leaf types (Figure 10.6.6a). Thus, the presence of the OxO transgene did not have any measurable effect on survival of caddisfly feeding on chestnut leaves.



*Figure 10.6.6a. Aquatic insect (caddisfly, *Frenesia difficilis*) survival on various leaf types. Qing CC = Chinese chestnut; WB 275 = WB275-27, non-transgenic American chestnut isogenic to Darling 4; F1 Hyb = F1 offspring from Chinese x American chestnut; 2G3 = marker-only transgenic American chestnut event AN-2G3; Dar4 T0 = transgenic American chestnut event Darling 4; Dar4 NT Full Sib = T1 offspring of Darling 4 that did not inherit the transgenes; Dar4 T1 = T1 offspring of Darling 4 that did inherit the transgenes (see Section 10.1 for descriptions of all the transgenic events). Green circles indicate OxO-expressing transgenic lines; blue circles indicate non-transgenic American chestnut controls.*

Other studies have examined effects of transgenic Bt (insect-resistant) crop plant material on aquatic insects. Even for Bt proteins, used specifically for their insecticidal properties, many of these studies are inconclusive or contradictory in terms of effects on stream invertebrates (Chambers *et al.*, 2010, Venter and Bøhn, 2016). One study that looked at transgenic deciduous tree (poplar) leaf effects on stream invertebrates (Axelsson *et al.*, 2011) surprisingly observed increases in insect abundance on Bt leaf litter compared to wild-type litter. Thus the lack of

detrimental effects against aquatic insects from OxO transformation, which does not impart known direct insecticidal mechanisms, is not surprising.

10.6.7 Terrestrial insect herbivory on legacy event transgenic chestnut leaves

Terrestrial insect herbivory has also been tested on transgenic American chestnut leaves in laboratory conditions and in permitted field plots. Tests directly involving Darling 58 chestnuts are described in Section 9.1.3; those on older OxO-expressing events are presented here.

A study conducted by Post and Parry (2011) compared the laboratory feeding behavior of three insect species on foliage collected from three chestnut types: Wirsig transgenic American chestnut (Section 10.1), unrelated wild-type American chestnut, and Chinese chestnut (cv 'Dalton'). (Wirsig is referred to as "LP28" in this study). Gypsy moth larvae were fed foliage from all three chestnut types. Polyphemus moth (*Antheraea polyphemus*) and fall webworm (*Hyphantria cunea*) larvae were fed six different foliage treatments: foliage from uninoculated trees of the three chestnut types, and separately, foliage from trees of all three types that had previously been inoculated with the chestnut blight fungus. All insects were weighed before and after feeding, and the relative growth rate was used as the response variable.

In the comparison between inoculated vs. uninoculated trees, relative growth rate of the insects (Polyphemus moth and fall webworm) did not differ significantly for any of the three tree types individually, or for all tree types combined. For Polyphemus moth and fall webworm, no differences were found between insects fed on transgenic vs. non-transgenic chestnut leaves. For gypsy moths, growth of larvae fed on Wirsig leaves was significantly faster (16%; $p < 0.012$) than growth of larvae fed on wild-type leaves. If gypsy moth growth is consistently faster on OxO-producing chestnuts than non-transgenic chestnuts, this could potentially indicate a novel pest risk by enhancing gypsy moth outbreaks. However, it is not possible to determine whether the differences in gypsy moth growth found in this study were due to the presence or expression of OxO in Wirsig, or simply a genotype difference between the two American chestnut lines. Wirsig trees used in the study were clonal, while the wild-type trees comprised an unrelated cohort of full siblings. WB275-27 trees, the non-transgenic clonal line isogenic to Wirsig, were not used as controls. Similar differences due to genotype have previously been observed in gypsy moth feeding on unrelated clones of aspen (Post and Parry, 2011), and one of the authors on this study has subsequently observed differences in insect herbivory between unrelated non-transgenic American chestnuts (Parry, personal communication). Additionally, Wirsig was later found to express OxO at very low levels such that blight tolerance in this event is essentially negligible (Section 10.1), so any differences observed are not likely due to OxO expression, and are not likely applicable to Darling 58.

A series of tests were later conducted to assess interactions between various chestnut lines, insect herbivores, and biocontrol parasites/parasitoids (Brown, 2016; Brown, 2017). Tests including Darling 58 leaves are described in Section 9.1.3, but there are additional data involving Darling 4 that are relevant to plant pest risk considerations which will be presented here.

In the summer of 2016, a study was performed on parasitoid activity on gypsy moth larvae feeding on seven chestnut lines in a common garden (Brown, 2016; Brown, 2017). Plants can respond to insect herbivory by releasing volatile compounds; these compounds can attract parasitoid

enemies of the herbivorous insect. One goal of this study was to detect any changes to parasitoid activity that could potentially result from disruption of this relationship by the insertion of a transgene.

Laboratory-reared sentinel larvae in the second instar were placed on 37 chestnut trees (equal replication was not possible due to tree mortality) of seven types: Chinese chestnut ('Cropper'), Backcross hybrid (BC3F3, Section 3.3.2), American chestnut seedlings (Zoar seed lot from Gowanda, NY) American chestnut produced from tissue culture (WB275, isogenic to the transgenic lines in this study), and three transgenic lines produced from tissue culture (Darling 1, Darling 4, and Darling 5; Section 10.1). Fifty larvae were placed on each tree, and after 15 – 25 days, larvae were collected and examined for parasitoid activity. Individual larvae were coded as either 1 (parasitized) or 0 (not parasitized), and effects of tree type were compared using generalized linear models (GLMs) and Tukey's HSD in R (R Core Team, 2014).

There were no significant differences in parasitism of gypsy moth larvae that had been deployed on any of the field-grown tree lines (Figure 10.6.7a), suggesting that the OxO transgene does not attenuate or amplify the parasitoid response of host trees.

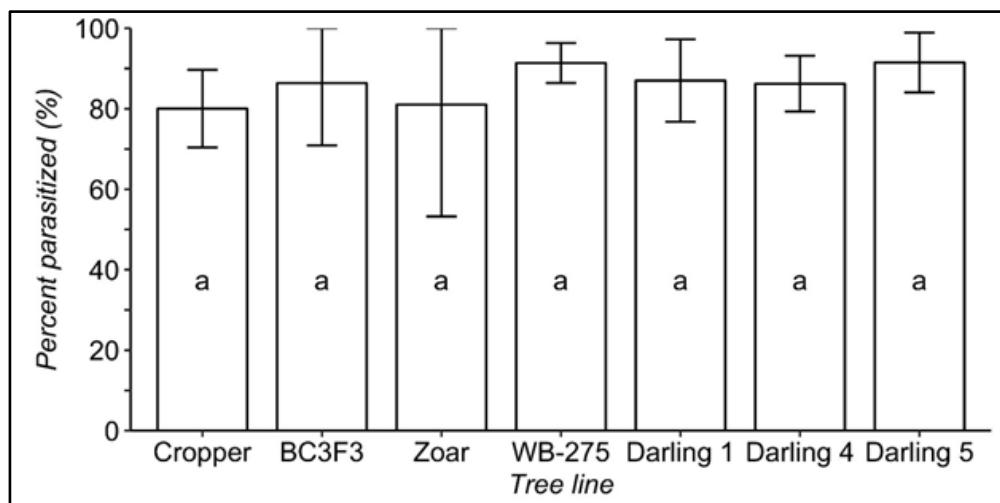


Figure 10.6.7a. Mean percent parasitism of collected sentinel caterpillars by tree line. Bars sharing the same letters are not significantly different ($p > 0.05$; Tukey HSD). Error bars are 95% CI. Cropper = Chinese chestnut, BC3F3 = backcross chestnut, Zoar = wild-type American chestnut seed lot, WB275 = WB275-27, isogenic non-transgenic American chestnut line, Darling 1, 4, 5 = transgenic American chestnuts (Section 10.1). From Brown (2017).

10.6.8 Tadpoles and chestnut leaf litter

Note: portions of the following are excerpts from Goldspiel *et al.* (2019).

In the spring of 2017, tadpole development and survival were observed in the presence of chestnut leaves; this simulates an interaction that might take place in vernal pools, which are temporary ponds that form in deciduous forests. Leaf species can have a significant impact on tadpole growth and survival (Stoler and Relyea, 2013; Earl *et al.*, 2014), and the loss of the American chestnut specifically may have had profound effects on the aquatic component of its ecosystems (Ellison *et al.*, 2005). Therefore potential effects of novel restoration material on wetland ecosystems are an important consideration for forest management or habitat

restoration, and represent a unique means of evaluating potential plant pest risks that could manifest themselves in these environmentally sensitive habitats.

Experimental setup (Figure 10.6.8a) consisted of a series of 1-quart jars each containing 0.8 g of crushed dried leaves (see below), 800 mL of dechlorinated water (changed weekly), and a single wood frog tadpole (*Lithobates sylvaticus*). All tadpoles were collected approximately one week after hatching, from a vernal pool near Tully NY, with authorization from the ESF Institutional Animal Care and Use Committee (Protocol 170401). Half of the jars for each leaf type received supplemental food (15 mg of mixed fish food flakes + rabbit chow) along with leaves, and supplement-only (no-leaf) controls were also included. 195 total jars were included: 15 replicated blocks * (Six leaf types * two supplement treatments + one no-leaf control). Observations included daily mortality and weekly growth and development measurements.

Tadpole sizes for growth comparisons were calculated from photographs of each tadpole, taken weekly with the tadpole in a shallow dish against a 1mm grid background, using the *Measure* tool in ImageJ software (Rasband, 2017). Development was recorded weekly in terms of a series of external limb characteristics known as the Gosner Stage (Gosner, 1960), which in this study ranged from stage 26 (rear limbs barely visible as protruding buds) to 42 (front limbs emerged; metamorphosis considered complete).



Figure 10.6.8a. Experimental setup for tadpole/chestnut leaf litter study (Goldspiel et al., 2019). 195 jars were placed on tables in a randomized block design, each jar contained 800 mL dechlorinated water, one leaf litter treatment, and one wood frog (*Lithobates sylvaticus*) tadpole. Inset photo is a single tadpole with transgenic chestnut leaf litter in a jar.

Leaf types in this test included transgenic and non-transgenic (full-sibling) American chestnuts, Chinese chestnut, F1 hybrid (American x Chinese) chestnut, American beech, and sugar maple. Beech and maple represent native trees in NY that are currently common in areas with vernal pools. Transgenic leaves were from T1 offspring of Darling 4 events, non-transgenic controls were from full-sibling trees of the same crosses that did not inherit the transgene. All leaves were collected directly from trees in or near a single permitted release site in the fall of 2015 and air dried at room temperature for approximately 18 months.

No-leaf control tadpoles showed high overall mortality, so maple leaves were considered the control treatment for comparison, since these trees are prominent in NY deciduous forests near

where this study took place. The only leaf type that was significantly detrimental to tadpole survival compared to sugar maple was American beech (Figure 10.6.8b). There were no significant differences in tadpole survival among chestnut leaf types, or between chestnut and maple controls.

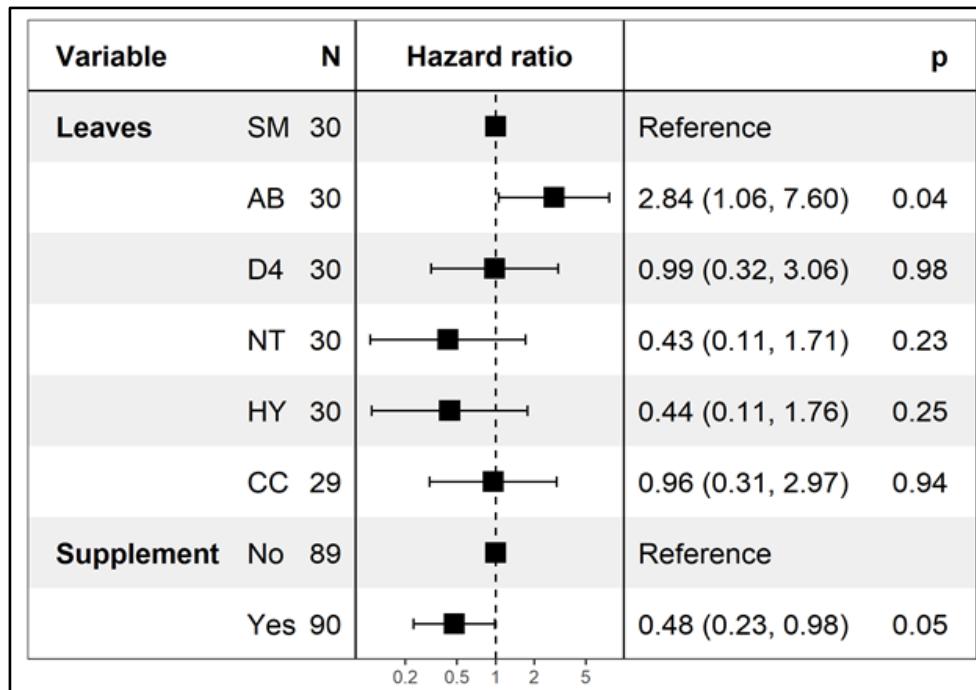


Figure 10.6.8b. Forest plot of Cox proportional hazard model contrasting relative tadpole mortality rate (i.e., hazard ratio) by leaf type and supplement. Hazard ratio values greater than 1.0 indicate an increased mortality risk relative to reference conditions (sugar maple, no supplements). SM = sugar maple, AB = American beech, D4 = transgenic American chestnut, NT = non-transgenic American chestnut, HY = F1 hybrid chestnut, CC = Chinese chestnut. From Goldspiel et al. (2019).

Tadpole development rates (Figure 10.6.8c, top) were also similar for most leaf types, with one notable exception: in jars that did not receive supplemental food, tadpoles developed significantly faster in the presence of American chestnut leaves, whether or not they were transgenic, compared to maple and other leaf types. In the presence of supplemental food, tadpoles exposed to American chestnut leaves (both transgenic and non-transgenic) developed slower than those with Chinese and hybrid chestnut leaves, but neither of these groups were significantly different than the maple reference type. Mean time to metamorphosis was also calculated for tadpoles in each leaf treatment (not shown). As with development rate, there were significant differences between transgenic chestnut and hybrid or Chinese chestnut, but no differences between transgenic chestnut and non-transgenic American chestnut or sugar maple. Tadpole growth rates (Figure 10.6.8c, bottom) were also similar among treatments, again with the exception of faster growth on American chestnut (both transgenic and non-transgenic) in non-supplement treatments.

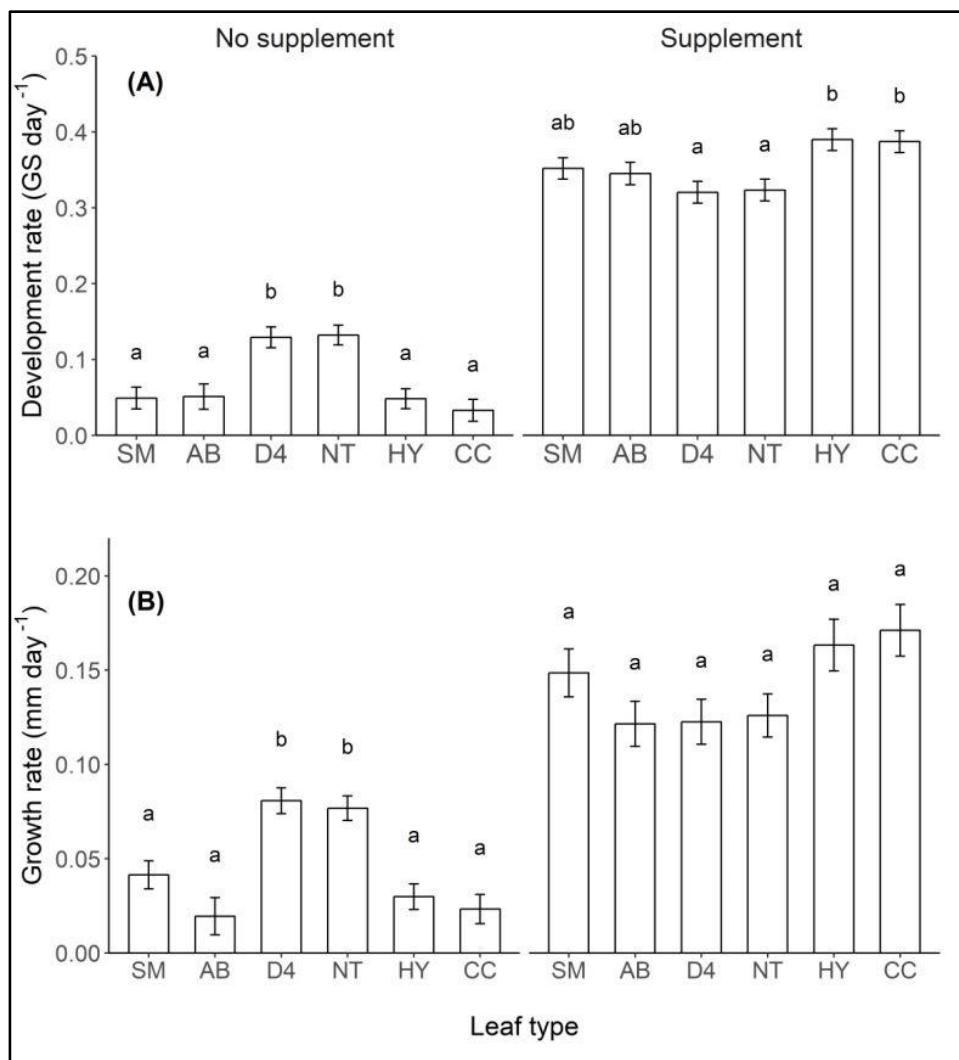


Figure 10.6.8c. Mean tadpole development rates (Gosner stage/day, upper charts) and growth rates (mm/day, lower charts) in different litter and supplement treatments from the start of the experiment to metamorphosis (or day 60). Day was log transformed for growth data. See Figure 10.6.8b legend for abbreviations. Treatments identified by the same lowercase letter are not significantly different from each other according to Tukey's pairwise comparison ($p > 0.05$). Error bars indicate ± 1 standard error of the mean. From Goldspiel et al. (2019).

Overall, these results provide no strong evidence that transgenic American chestnut leaf litter negatively affects tadpole performance. Tadpoles performed similarly in both transgenic and non-transgenic American chestnut treatments, indicating that the OxO transgene and the transgene insertion process are unlikely to present novel risks for anuran larvae. Further detail and discussion can be found in (Goldspiel et al., 2019).

10.6.9 Responses to other pests

As with Darling 58 (Section 9.2), Darling 4 and other events have been extensively propagated, cultivated, planted, and observed for many years, always in close proximity with non-transgenic isogenic lines (WB275-27, in the case of Darling 4). Targeted pest surveys were not conducted beyond blight inoculations, but we have occasionally observed incidental pest or pathogen

infections, primarily generalist herbivores such as aphids and mealybugs, or opportunistic pathogens such as powdery mildew. Field-planted transgenic trees have occasionally experienced chewing from rodents such as voles. We have consistently observed that OxO-expressing chestnuts including Darling 4 experience these infestations at similar rates as non-transgenic controls, and resulting damage is not visibly different. For example, in a greenhouse table or field plot containing both transgenic and non-transgenic chestnuts, we have never been able to identify transgenic individuals based on pest/pathogen infection severity (other than blight). While all of these observations are informal and anecdotal, they have been entirely consistent with regards to a lack of visible differences between transgenic trees and isogenic non-transgenic controls.

11.0 Unique considerations for transgenic chestnuts

The Darling 58 tree described in this petition is unique in a number of ways from other plants previously submitted to APHIS for a determination of nonregulated status. It is a long-lived forest tree intended for non-profit use in environmental restoration, not a patented crop plant intended for managed commercial use. It is intended to outcross with wild relatives, which would allow the transgene to introgress into wild populations. It was intentionally not developed in a homozygous transgenic state, which will allow enhanced diversity via outcrossing and continual production of fully wild-type offspring. Potential benefits of this introgression include allowing a currently-threatened native tree to once again thrive in its natural range, which could restore ecosystem services and cultural values that have been essentially absent for a century, while still preserving non-transgenic genotypes.

This is in stark contrast to annual agricultural crops, which are intentionally contained and harvested within strict field boundaries and often managed to *prevent* outcrossing. Profitable crops understandably make up the majority of plants that have heretofore been granted nonregulated status by APHIS, but emerging biotechnologies are putting a variety of non-agricultural applications within reach (Corlett, 2017; Piaggio *et al.*, 2017). We hope consideration of transgenic Darling 58 American chestnuts (and future petitions for similar restoration purposes) will be understood in the context of alternative options, which may include traditional breeding, planting alternative species, changing the pathogen using hypoviruses or other biocontrol agents, other forest management measures, genetic engineering, and the choice to do nothing regarding a specific threat. All of these options carry potential risks or benefits, and they should be evaluated accordingly by regulators, landowners, stakeholders, and other decision-makers.

11.1 Paradigm shift: intentional introgression into wild populations to benefit the environment

American chestnuts as a species have several unique attributes that make them uniquely suited to be the first application of genetic engineering for potential wild release or environmental restoration. They are relatively slow to spread, not weedy, easy to identify, slow to flower, and take years to produce seeds. They are easy to exclude from an area if someone desires to do so (Section 11.6). There have already been numerous unregulated plantings in the US of non-native chestnut species, hybrids, and irradiated lines (Sections 2.1.2 and 3.3), so the concept of restoring forests to an idealized, fully native, unmodified pre-blight condition is not realistic. The idea that this may be the first transgenic organism in a natural setting is being challenged by recent research suggesting that about 7% of all dicot species may have been naturally transformed in their evolutionary past with DNA from *Agrobacterium* (Matveeva and Otten, 2019).

American chestnuts are also considered functionally extinct, surviving primarily as stump sprouts that rarely reach sexual maturity in nature, often confined to small isolated remnant populations (Section 2). So even in the extremely unlikely event there is some unforeseen detrimental effect of releasing transgenic trees in forests, there is a low risk of harm to existing American chestnut populations, and surviving blight-tolerant American chestnuts would be easy to identify. On the other hand, American chestnuts are not totally extinct; the remaining genetic diversity in surviving

trees should allow meaningful restoration of diverse populations given adequate time and outcrossing effort (Section 11.2).

American chestnut might be the first environmental restoration-focused transgenic organism submitted for regulatory consideration by APHIS, but others will likely follow. Many other trees and valued wild plants are threatened by pests and diseases, and that number will likely continue to grow with increasing pressure from both rising temperatures and invasive species (Dukes *et al.*, 2009; Roy *et al.*, 2014; Millar and Stephenson, 2015; Fei *et al.*, 2019). A recent National Academies of Science, Engineering, and Medicine (NASEM) study, “The Potential for Biotechnology to Address Forest Health,” highlights the immediate relevance of these topics to government and other audiences (NASEM, 2019). During one presentation to this NASEM panel, a representative from the US Fish & Wildlife Service’s Forest Ecology Working Group shared that their group doesn’t necessarily promote restoration of single tree species, or condone any particular means of producing disease-resistant trees. However, tree restoration (by hybridization, genetic engineering, or other methods) might be of particular value to their agency if it facilitates broader habitat restoration and enhances ecosystem function (Hunter and Alanen, 2018). A report has also been published by the International Union for Conservation of Nature (IUCN) assessing “Synthetic Biology and Biodiversity Conservation” (Redford *et al.*, 2019), which, like the NASEM study, highlights the urgency of conservation, uses transgenic American chestnut as a case study, and recognizes that other applications of biotechnology for conservation will likely be available or in use soon.

11.2 Intended distribution strategies for Darling 58 American chestnuts

It is clear that eastern US forests are substantially different now than they were in the early 1900s when chestnut blight was first spreading. However, that does not mean that American chestnut restoration should be abandoned. On the contrary, restoration efforts such as American chestnut reintroduction may actually be synergistic toward achieving broader environmental goals involving forest health that are especially relevant today. TACF and others have been seriously considering the implications of chestnut restoration for decades (Hill, 1994; USFWS, 2006; Jacobs, 2007; Pinchot, 2011; Dalgleish and Swihart, 2012; Fei *et al.*, 2012; de Bruijn *et al.*, 2014; Gustafson *et al.*, 2017; Gustafson *et al.*, 2018). Based on our conclusion that transgenic American chestnuts are not substantially different from non-transgenic alternatives apart from blight tolerance (Sections 8 and 9), ecological implications of chestnut restoration should be similar regardless of how blight-tolerant material was produced.

Wild plantings of potentially blight-tolerant material produced by unregulated methods have been attempted for several decades in various locations (Diller *et al.*, 1964; Dietz *et al.*, 1978; Section 3.3). As a result of these and other efforts, many types of chestnuts are already present both within and beyond the American chestnut’s original range: these include radiation-mutated American chestnuts, hybrid chestnuts produced from several different species, introduced Chinese chestnuts, and various types of chestnuts infected with natural or lab-generated hypovirulent blight fungi. Thus restoration with transgenic Darling 58 American chestnuts would not represent a fundamentally new introduction of potentially blight-tolerant chestnuts to existing habitats.

If nonregulated status is granted, we currently envision that distribution and planting of Darling 58 American chestnuts and their progeny will take place within three distinct programs: long-term research and demonstration plantings (some of which have been started under permits), a relatively small-scale public horticultural program for which trees and/or pollen will be available soon after regulatory approval is granted, and a larger-scale public restoration program that will likely involve years of outcrossing and production before substantial plantings take place. Research and observations will be a collaborative effort between ESF, TACF, and other researchers, while both of the public distribution programs (horticultural and restoration) will be primarily overseen by TACF.

Collaborative, long-term field trials have already been initiated with support from the USDA-NIFA BRAG program (Proposal number 2018-02688) and public donations. Initial plantings have taken place under APHIS permits, with the intent of observing long-term growth and natural dispersal when/if a determination of nonregulated status is granted. These research and demonstration plots consist of both open field and shelterwood environments located in three states, with the goal of comparing offspring of Darling 58 American chestnut to traditionally produced chestnut trees over several decades.

Within the public horticultural distribution program, propagules (which may include pollen, scion wood for grafting, seed, and seedlings) will initially be offered to individual supporters of ESF's American Chestnut Research and Restoration Project, as well as certain historical, cultural, environmental, or educational institutions. (Wider public distribution will follow depending on availability of propagules.) Recipients will be free to plant and propagate the trees they receive with no obligations, but as "citizen scientists" they will be encouraged to record and monitor plantings and report observations back to ESF or TACF researchers. Due to the limited genetic diversity of this early distribution (primarily T1 and T2 generations), these trees will be considered horticultural trees, though recipients may choose to help diversify offspring by planting them near wild-type chestnuts in forest settings, or by intentionally cross-pollinating them with wild-type trees. This horticultural distribution may include up to thousands of propagules, but will ultimately take place on a small scale relative to the historic population and range of American chestnuts, which was estimated to include over 3 billion trees.

In parallel with distribution of horticultural trees, a long-term restoration breeding plan is being developed in collaboration with TACF (Steiner *et al.*, 2017; Westbrook *et al.*, 2019a). The plan involves outcrossing Darling 58 trees with diverse individuals from many of the surviving small remnant American chestnut populations, resulting in enhanced genetic diversity and adaptability among the progeny. Though these surviving wild-type individuals are susceptible to blight, enough survive to flowering age (especially with care or intervention, see Section 3.3.1) to allow pollination to take place. Additionally, many individuals and TACF chapters are actively caring for and pollinating wild-type American chestnuts in small plots throughout the original range, so these cross-pollination efforts are not only feasible, but already underway with non-transgenic individuals (Fitzsimmons, 2017; Section 2.1.1). Based on computer simulations of various introgression scenarios, more than one generation of outcrossing to American chestnut will be required to "dilute" the limited diversity found in the single genetic background (Ellis), minimize inbreeding, and expand effective population size (Westbrook, 2018; Westbrook *et al.*, 2019a). Additional events beyond Darling 58 may be produced and submitted for evaluation by APHIS in

the future to further increase genetic diversity (especially alleles nearby the transgene insertion site) during the diversification breeding.

The extended time scale of this diversification effort will allow results and feedback from long-term research plots and early horticultural plantings to be incorporated before large-scale restoration plantings are initiated. This plan will be coordinated by TACF and likely carried out by TACF state chapters, state or commercial tree nurseries, and other collaborators throughout the range of the American chestnut. Careful attention will be given to predicted future habitat suitability (Barnes and Delborne, 2019), and incorporating appropriate genetic diversity from surviving American chestnuts from various parts of their native range. The products of this breeding program may ultimately be introduced to forests on a larger scale (in coordination with public and private forest land managers), with the aim of establishing a self-sustaining, diverse, resilient, blight-tolerant population of American chestnut trees.

11.3 Public engagement, transparency, and the history of Darling 58 American chestnuts

The bioengineering of American chestnut for blight tolerance was initiated by the public and the project team has frequently engaged (and been engaged by) the public ever since. This started in 1989 when three leaders of the newly established New York chapter of The American Chestnut Foundation (NY-TACF), Herb Darling and Stan and Arlene Wirsig, approached Dr. Maynard and Dr. Powell at ESF with a proposal (Powell *et al.*, 2019). TACF as a national organization was proceeding with a backcross breeding program started in 1983, but the NY chapter members wanted to take a parallel path using the then-new technology of genetic engineering. So began the American chestnut bioengineering project in 1990, as a close collaboration between this public organization and ESF (Neumann, 2018). Venues such as annual meetings, quarterly newsletters, shared tours or presentations, and chestnut planting events allow continued engagement between these groups in the form of reporting research progress, trying new chestnut planting and growing techniques, providing and soliciting feedback, and sharing mutual encouragement.

It has always been the philosophy of ESF and NY-TACF for the American chestnut project to be as transparent as possible and listen to the public's responses (Dougherty, 2016). This has taken place via various means such as multiple field and lab tours each year, holding public demonstration plantings (under USDA permits) at the New York Botanical Garden and on the ESF campus, inviting the public to help with research plantings, engaging the public at NY State and local county fairs, and frequent presentations (with Q and A sessions) to audiences of up to 350 people at both professional and public meetings. Notable presentations include a TEDx talk sponsored by National Geographic with more than 136,000 views to date¹¹, K-12 educational programs, presenting the project in college classes, multiple webinars with both the Fish and Wildlife Service and the National Academies of Science, and hosting a Reddit "Ask Me Anything" (1,240 participants, 89% up-voted). We have engaged nonregulatory governmental agencies such as the U.S. Forest Service, the Fish and Wildlife Service, National Parks Service, and the New York State Department of Conservation. We also have interacted with local parks, private conservation

¹¹ "Reviving the American forest with the American chestnut" | William Powell | TEDxDeExtinction. YouTube, uploaded by TEDx Talks, April 7, 2013, Available at: youtu.be/WYHQDLCmgyg

groups, and other NGOs such as the Ozark Chinquapin Foundation (Section 11.4.2). We have reached out on multiple occasions to Haudenosaunee (Onondaga Nation) leaders in New York State to both present our project and receive feedback.

We also share and engage via written, web, and broadcast media whenever possible. Articles by research team members outside of academic journals have been featured in The Conversation (Powell, 2016), Scientific American (Powell, 2014), and the Washington Post WorldPost (Newhouse, 2018). These articles and other events have in turn resulted in many local, national, and international articles, including PBS TV and radio, CBC TV and radio, WIOX community radio, The Atlantic, The Wall Street Journal, National Geographic, Scientific American, The Economist, Smithsonian, Ensia magazine, the Pacific Standard, and the LA Times. Many of these articles have received largely positive comments from public readers. Another unsolicited Reddit open discussion involved 35,415 participants, with 90% up-voted. ESF has hosted two crowdfunding campaigns for chestnut research that together yielded 1,453 donations totaling \$709,000, with donors hailing from forty-eight states and six countries. An extensive group of webpages and social media groups has been developed¹². As of spring 2020 these social media outlets have a combined total of more than 4,300 members/followers, and regularly result in active discussions, interactions with the research team, and feedback to the project.

This public engagement has gone beyond simply being transparent and reporting progress. Public feedback has directly informed many aspects of the chestnut research project, including its initial establishment, which genes to use (or avoid), the decision to focus only on blight tolerance rather than trying to make a “super tree”, which environmental compatibility tests are most relevant to the public interest, and the decision to avoid limitations on distribution through control of intellectual property rights. The project has always welcomed and incorporated public feedback while acting on scientifically sound advice. This public engagement will necessarily continue into the future, because restoration of the American chestnut (or other uses of bioengineering for broader environmental goals) is not a decision to be made only by researchers or regulators, but also with the input of local communities such as interested public groups and individuals who will be planting and enjoying these trees (Kofler *et al.*, 2018).

11.4 Examples of public support and responses

In addition to developers, other scientists, and regulators, there are a variety of public individuals or groups (sometimes collectively called “publics” or stakeholders) who have specific interests in, or concerns about, some aspect of chestnut restoration. Their responses and opinions can be complex and multifaceted, even within single groups, but we have attempted to summarize some of these responses below, based on social media engagement, personal communications, and publications.

It is important to note that many public concerns about genetically engineered chestnuts are either not specific to genetic engineering (i.e. would also apply to backcross hybrid chestnuts), or

¹² Project home page available at: <https://www.esf.edu/chestnut/>
Facebook pages available at: <https://www.facebook.com/groups/esfchestnut/> and
<https://www.facebook.com/americanchestnutgroup/>
Instagram page available at: https://www.instagram.com/american_chestnut_project/?hl=en
Twitter page available at: <https://twitter.com/chestnutPowell>

not specific to chestnuts (i.e. the ultimate concern is actually about application of GE to other tree species unrelated to chestnuts or restoration efforts). Given the ideological nature of some such claims, some skepticism will not likely be satisfactorily addressed with data. As mentioned elsewhere, vocal ideological opposition has been a small minority voice in conversations about genetic engineering for chestnut restoration. A National Research Council report (National Research Council, 2015) reminds us that "...the presence of small groups of passionate stakeholders does not suggest that the wider public is in a state of division about GMOs." More specific concerns (many of which are addressed in this petition) include an assumed lack of genetic diversity (Section 11.2), potential nontarget effects (Sections 9.1 and 10.5), and unwanted introgression with wild populations (Sections 2.2.2, 2.4, 11.2, and 11.6).

If changes to the chestnut genome are a concern, it should be noted that both hybrid breeding and mutagenesis can result in far more genomic changes than genetic engineering (Schnell *et al.*, 2015; Anderson *et al.*, 2016). Other concerns may be conflated with agricultural traits like herbicide tolerance or insect resistance, or with motivations of for-profit agricultural companies, none of which are directly relevant to Darling 58 American chestnuts. When considering the merits or risks of Darling 58 American chestnuts, we encourage people to intentionally define the scope of their hopes or concerns, and to be aware of issues that may apply equally to non-GE chestnuts, or non-chestnut GE products.

11.4.1 Chestnut enthusiasts and general audiences

As described in Section 11.3, the entire effort of producing a transgenic American chestnut tree was initiated not by scientists, but rather by a group of chestnut enthusiasts. These generous and dedicated individuals were founding members of the non-profit NY-TACF, which has followed and supported the ESF research effort ever since. This long-term coordination and transparency between academic (ESF) and public (NY-TACF) has resulted in a unique collaboration leveraging the strengths of both types of groups, which could perhaps be a model for other projects seeking to employ biotechnology for non-profit purposes. Beyond the NY chapter, TACF as a national organization has also shown increasing acceptance and support of the transgenic chestnut, incorporating biotechnology into potential long-term restoration plans (Section 11.2; The American Chestnut Foundation, 2017; Steiner *et al.*, 2017).

As described in Section 11.3, ESF chestnut researchers and members of NY-TACF regularly give presentations, interviews, tours, workshops, and other shared interactions with various members of the public. These include venues and audiences who don't necessarily have a specific pre-existing interest in chestnut restoration, such as public libraries, garden clubs, woodworking clubs, forest landowner groups, scientific conferences, and newspaper or magazine reporters. Even without previous chestnut interests, a single response is overwhelmingly prevalent after people hear about Darling 58 American chestnuts: "How soon can I have one to plant?" Even audience members who state that they're skeptical of commercial agricultural biotechnology often acknowledge that they would accept genetic engineering for non-profit or environmental restoration purposes. Additionally, articles in non-academic media outlets (e.g. Powell, 2016; Faubel, 2018; Newhouse, 2018) have yielded many positive comments from readers and even positive responses to negative comments, which is not always the case for popular press articles on genetic engineering.

11.4.2 Chinquapin enthusiasts

The Ozark chinquapin (Section 2.1.1) is a tree-form relative of the American chestnut that has also been effectively wiped out by chestnut blight (Paillet, 1993). As with the American chestnut, there are enthusiastic supporters of this tree who would like to see it restored to its former place in the local ecosystem (Thomas *et al.*, 2007). This is being pursued and managed primarily by the Ozark Chinquapin Foundation (ozarkchinquapinmembership.org), currently using traditional breeding and selection processes (Langellier, 2019). This group specifically wishes to avoid breeding with non-native (Asian) chestnuts, and has reported preliminary success in achieving blight resistance through traditional breeding (Bost, 2019). However, they support ESF's efforts with OxO-expressing American chestnut, and they also support the use of genetic engineering for research on Ozark chinquapin. Progress toward restoration of either *C. dentata* or *C. ozarkensis* would be mutually encouraging for both of their respective Foundations.

11.4.3 Indigenous groups

The ESF chestnut research team has worked with ESF's Center for Native Peoples and the Environment to reach out to local Haudenosaunee leaders on multiple occasions, including once in conjunction with the New York Department of Environmental Conservation. The purpose was to maintain transparency by sharing information and receiving feedback. Historical interactions between university researchers and indigenous groups have been fraught with power imbalances and sometimes hidden agendas, but these chestnut meetings seemed to be met with cautious optimism by both ESF and Onondaga representatives. As with general public opinion surveys (Section 11.4.4), two recent articles focused on Haudenosaunee people in New York have reported a range of responses to chestnut restoration, including both acceptance and skepticism regarding transgenic trees, skepticism about planting or breeding with non-native chestnuts, and concern about active restoration efforts in general (Barnhill-Dilling and Delborne, 2019; Rosen, 2019).

The timing of the American chestnut's decline largely coincided with major cultural disruptions to Haudenosaunee communities in and around New York. Efforts are underway to restore cultural practices such as language, diet, and traditional ecological knowledge, and it is possible that such cultural restoration may harmonize with ecological restoration efforts such as American chestnut reintroduction (Kimmerer, 2011; Barnhill-Dilling and Delborne, 2019). Additionally, Dr. Percy Abrams (Syracuse University) and Neil Patterson (ESF) have been researching cultural and language learning, including working with local representatives to establish indigenous words for modern concepts like "gene" and "genetic engineering" (Patterson presentation to NY DEC and ESF, October 2017). Language and indigenous terminology is especially important to the Haudenosaunee for cultural relevance and understanding of new (or lost) concepts, so language development and teaching should help facilitate informed decision-making about potential chestnut re-introduction and other restoration projects. In part to help re-familiarize people with this historical food source, NY-TACF and ESF regularly share wild-type chestnuts for both food and planting, and blight-tolerant trees will also be made available if groups or individuals are interested in growing them to learn more about their potential integration into traditional practices.

11.4.4 General public (opinion surveys)

Several large-scale empirical surveys have been conducted on broad public opinions regarding the use of biotechnology for forest health (Friedman and Foster, 1997; Gamborg and Sandøe, 2010; Hajjar *et al.*, 2014; Hajjar and Kozak, 2015; Nonić *et al.*, 2015; Fuller *et al.*, 2016; Kazana *et al.*, 2016; Needham *et al.*, 2016; Jepson and Arakelyan, 2017a; Jepson and Arakelyan, 2017b; Urquhart *et al.*, 2017). These surveys have taken place in the US, UK, Europe, and Canada, but the general consensus is similar: in the face of a concrete threat like chestnut blight, public acceptance of biotechnological solutions is similar to acceptance of traditional breeding or planting of non-native species, and biotechnological solutions are generally more favorable than doing nothing. For example, two surveys of Canadian residents (Hajjar *et al.*, 2014; Hajjar and Kozak, 2015) concluded that when faced with forest health threats due to climate change, approximately 50 – 60% of respondents would support either planting non-native trees or trees developed using biotechnology, while only 35% would accept doing nothing in response to the climate threats.

In the survey most specifically relevant to American chestnuts, Needham *et al.* (2016) asked members of the US public ($n = 278$, weighted by Census, not selected by interest or knowledge of chestnuts) about their support interventions for addressing chestnut blight, and a majority of respondents said they would vote for transforming chestnut with a wheat gene (61%), while slightly less than half would vote for breeding American chestnuts with non-native trees from Asia (44%). The same work by Needham *et al.* (2016) is also summarized by Delborne *et al.* (2018) and a recent National Academies study (Chapter 4 in: NASEM, 2019). This National Academies study also reviews some of the other public survey data (listed above) in more detail.

More generally, two surveys were recently conducted in the US about public attitudes regarding the use of biotechnology for wildlife conservation (Kohl *et al.*, 2019; Thresher *et al.*, 2019). While these are not about trees specifically, underlying public attitudes for environmental applications of biotechnology may be relevant. Kohl *et al.* (2019) reported that most people perceive risks associated with gene editing technologies in general, especially when used to eliminate or decrease populations of pests or invasive species, and noted concerns that gene editing “could be used for the wrong purposes.” However, they report that “a relative majority agreed applications to improve survival in endangered species would be morally acceptable” (Kohl *et al.*, 2019). Thresher *et al.* (2019) focused specifically on stakeholder responses to the potential use of recombinant DNA technologies to manage Sea Lamprey in the Great Lakes. They reported that both “stakeholders” (e.g. biologists or fisheries managers already involved in lamprey management) and the local public fishing community strongly supported initiating research toward recombinant techniques, and would subsequently support *in situ* implementation if risks (especially non-target impacts) were shown to be low (Thresher *et al.*, 2019).

Collectively, these survey data reinforce general responses frequently received by ESF chestnut research team members: a minority of audience members are skeptical about breeding with Asian chestnuts, another minority is skeptical about genetic engineering, but in our experience most people simply like the idea of restoring American chestnuts by whatever means are safe and effective.

11.5 Lack of association between OxO and gluten

While gluten sensitivity may not be considered a plant pest risk factor, it is a major concern for many people, especially given that chestnuts are a unique gluten-free source of carbohydrates that can be used for baking, brewing, and other food products. A common question when people hear about the use of a gene from wheat in chestnuts is whether this will be a problem for people with Celiac disease (CD) or other gluten sensitivities. The director of ESF's chestnut project, Dr. Powell, has CD and was personally interested in answering this same question before using the OxO gene. The answer is no: OxO isn't related to gluten. Gluten is comprised of storage proteins found in a protein superfamily called prolamins (Thompson, 2001), which is entirely separate from the cupin superfamily containing GLP's such as OxO (Section 4; Radauer and Breiteneder, 2007). OxO is only one gene out of over 100,000 genes in wheat, most of which are unrelated to gluten.

As with potential concerns about toxicity or allergenicity (Sections 8.4.4 and 8.4.5), the presence of native OxO genes in gluten-free food crops such as sorghum, rice, and corn (Section 4.2) should ease concerns for gluten-sensitive individuals. A lack of association with Celiac-inducing peptides was confirmed using the University of Nebraska's AllergenOnline Celiac Database (version 2, released January 18, 2018¹³; Goodman *et al.*, 2016).

The AllergenOnline site (as of January 2019) describes the database search as follows:

"In addition to the Exact Peptide match, the linked Celiac Disease database also includes a FASTA algorithm to compare the query protein against 72 celiac inducing proteins that are the sources of the peptides and list of 69 published references supporting the inclusion of peptides and proteins in the database. Proteins lacking any identity match to the 1,013 peptides are not likely to trigger celiac disease, however it is possible that not all peptides that can trigger CD are known. Thus FASTA to the 68 proteins adds a level of safety. The FASTA comparison has not (yet) been validated sufficiently to set absolute thresholds of concern for celiac disease. However, preliminary searches with proteins from rice, sorghum, maize and other food sources that are considered safe for those with celiac disease allowed us to establish reasonably conservative guidelines. Identity matches of less than 45 percent over at least one-half of the FASTA aligned CD protein and those with an E score greater than 1×10^{-16} using this database are unlikely to present a risk of inducing celiac disease."

We queried the amino acid sequence of oxalate oxidase (Table 7.1.1a) against this database using both the Peptide Exact Match and Full FASTA searches. The Peptide Exact Match returned no matches (Figure 11.5a), and the Full FASTA returned no matches above 10% identity (well below the 45% threshold suggested by the developers). Databases at this site were also queried for keywords including "oxalate oxidase" and "oxo," again with no matches. Due to the lack of concerns about gluten in other OxO-expressing food crops, and the lack of database matches to known Celiac-triggering peptides, we conclude that oxalate oxidase as expressed in Darling 58 chestnuts should not be a concern for gluten-sensitive individuals.

13 Available at: www.allergenonline.org/celiachome.shtml

MGYSKTLVAGLFAMILLLAPAVLATDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFLFSSKLAKAGNTS
TPNGSAVTELDVAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPRATEIGIVMKGELLVGILGSLDSGNKLYSR
VVRAGETFLIPRGLMHFQFNVGKTEASMVVSFNSQNPGIVFVPLTLFGSNPPIPTPVLTALKALRVEARVELL
KSKFAAGF

Submit Query

>query

>query

MGYSKTLVAG LFAMILLLAPA VLATDPDPLQ DFCVADLDGK AVSVNGHTCK PMSEAGDDFL
FSSKLAKAGN TSTPNGSATV ELDVAEWPGT NTLGVSMNRV DFAPGGTNPP HIHPRATEIG
IVMKGELLVG ILGSLDSGNK LYSRVVRAGE TFLIPRGLMH FQFNVGKTEA SMVVSFNSQN
PGIVFVPLTL FGSNPPPIPTP VLTKALRVEA RVVELLKSKF AAGF

Your Search returned 0 results

No exact peptide matches found

Figure 11.5a. Screenshot from AllergenOnline Celiac Database website, confirming no matches for the OxO amino acid sequence compared to known Celiac-triggering peptides. Similar results from FASTA and text searches are not shown.

11.6 Options for controlling establishment of specific chestnut types

For various reasons, certain groups, municipalities, or individuals may be interested in restricting chestnut establishment to a single type, or precluding establishment of specific chestnut types on their land. Such chestnut types may include non-native Asian species or hybrids, transgenic, irradiated, or others (Section 3.3).

Regardless of the type of tree someone wishes to avoid or the reasoning behind these wishes, chestnut establishment can generally be prevented for at least several decades by simply *not planting that type of chestnut*. As described in Section 2.4, it is likely that at least the first several decades of chestnut restoration will depend on people intentionally planting trees and caring for them in order for reintroductions to be stable and successful (Gustafson *et al.*, 2017). Relatively slow rates of American chestnut introgression to new areas can also be inferred from post-glacial spread of trees in the eastern US and detailed dispersal records from pre-blight plantings outside the original American chestnut range, both of which are discussed in Section 2.4. Each of these sources has a high degree of uncertainty due to the limited locations or data available, and establishment may be faster on areas with site conditions particularly favorable to chestnut recruitment.

Population expansion following restoration plantings of blight-tolerant chestnuts was modeled by Rogstad and Pelikan (2014). Based on data from a small planting of American chestnuts in West Salem, WI (Section 2.4), they use the following parameters in their model for age-specific expected offspring per year per individual: zero offspring from trees aged 0 – 7 years, 0.05 offspring/year from 8-year-old trees, 0.13 offspring/year from 10-year-old trees, 0.2 offspring/year from 12-year-

old trees, 0.5 offspring/year from 17-year-old trees, and 4.9 offspring/year from trees aged 70 – 113 years (Rogstad and Pelikan 2014). This corroborates previous conclusions that dispersal will be very gradual, even from established chestnut trees.

Even if mature chestnuts are present nearby, slow natural colonization rates and frequent animal and pest pressure on seeds and seedlings (Clark *et al.*, 2014) suggest that chestnuts, regardless of type or transgene status, will not rapidly invade new areas (Cook and Forest, 1979). Chestnuts do not produce wind-dispersed seeds like cottonwood, blankets of seedlings like maple, or root sprouts like aspen. They do require at least two mature individuals nearby each other to form viable seeds (Section 2.2.2), so chestnut dispersal is relatively slow without human intervention. If further assurance is desired by individual land managers or groups, occasional (~yearly or less) monitoring should allow identification of volunteer chestnuts long before they flower, at which point they can generally be simply uprooted by hand, treated with herbicide, or mowed. A similar scenario already exists for commercial chestnut growers or breeders, who have to be aware of what (if any) flowering chestnuts are growing near their orchards to prevent pollination by unwanted species or hybrids (unintentional crosses with these non-transgenic trees can cause problems like male sterility and Internal Kernel Breakdown; Section 2.1.2). This type of control by growers has not been reported to be onerous.

Widespread deployment of restoration products like the Darling 58 American chestnut is not inevitable, even if nonregulated status is granted. Many researchers, practitioners, and individual chestnut enthusiasts would certainly like to see a blight-tolerant American chestnut tree successfully reintroduced to its former habitat (e.g. Section 11.4). But ultimately, successful restoration and widespread establishment depends on continued efficacy and safety of the restoration material, coupled with broad public interest and involvement, including official support from groups such as TACF and others (Appendix X). If some sector of the public (regionally, culturally, or nationally) decides not to plant a certain type of chestnuts in an area, that is their decision to make, and that type of chestnut is unlikely to rapidly introgress without assistance. Given the relatively slow natural colonization rates of American chestnut (Sections 2.2 and 11.2), areas that are not intentionally planted with blight-tolerant chestnuts will likely *remain* without chestnuts for decades or longer, much as they have for the past century.

One example of regulatory approval not resulting in subsequent US distribution is demonstrated by the virus-resistant C5 plum, which received nonregulated status from APHIS in 2007 and FDA and EPA approval by 2011 (Scorza *et al.*, 2013), but has still not been widely planted in the US. While much research and careful planning have already gone into potential chestnut restoration scenarios (Section 11.2), there is still a need for continued public engagement and feedback regarding planting plans and locations, and there will be many years to collect such feedback before large scale restoration plantings might occur. Such engagement has already guided research and development of Darling 58 American chestnuts (Section 11.3), and will continue to inform decisions moving forward.

11.7 Conclusions and considerations for other tree conservation interests

In addition to American chestnut, there is a long and growing list of other trees facing serious threats from invasive pests and diseases, some of which may threaten substantial portions of US forest biomass (Fei *et al.*, 2019). Notable examples around the United States include Emerald ash

borer, Dutch elm disease and elm yellows, Hemlock wooly adelgid, thousand cankers disease of walnut, western pine bark beetles, sudden oak death, and Rapid ‘Ohi'a death. Early work has been done on transgenic elm (Gartland *et al.*, 2005; Newhouse *et al.*, 2007) and ash (Palla and Pijut, 2015; Lee and Pijut, 2017), and more extensive research has been done on transgenic plantation-type trees like poplar (Klocko *et al.*, 2016; Strauss *et al.*, 2017), but to our knowledge none of these species have been submitted for regulatory consideration. Thus the transgenic American chestnut may represent an important first step in offering a safe and effective tool for addressing threats to forest health. Lessons learned from transgenic American chestnut research may also serve as a road map for the extension of advanced plant science, biotechnology, genomics, bioinformatics, gene editing, regulatory science, and breeding tools from annual crops to threatened wild plant populations (Jacobs *et al.*, 2013). In addition to potentially addressing forest health threats, continued research in these fields could create jobs, research and teaching opportunities, and economic growth.

Of course some forest health threats may be suitably addressed by traditional breeding, selection programs, sanitation/cultural practices, planting alternative species, or other management techniques (Woodcock *et al.*, 2018). Additionally, it will be essential to actively involve local communities and affected groups in decision-making about research programs, for directions such research should take, and for social considerations beyond issues of ecological merit or safety (Kofler *et al.*, 2018; Stirling *et al.*, 2018; Section 11.3). But if genetic engineering or other emerging technologies have the potential to safely and effectively address critical threats to forest health, it would be wise to consider these tools, and remember the possible negative implications to forests, ecosystems, and people if we choose to do nothing.

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Appendices

Appendix I. Field trial authorizations

Authorizations (permits and notifications) owned by ESF faculty (top) and research collaborators (below) covering interstate movement or confined release of Darling 58. No unusual occurrences, non-target interactions, or deleterious effects have been observed.

| USDA APHIS Authorization Number | Effective Date | Location | Purpose (for ESF Authorizations) | Summary of Darling 58 observations and results (for ESF Authorizations) |
|---|--------------------------------------|-------------|---|---|
| 19-065-101n, 18-068-101n | 4/10/2019, 4/1/2018 | NC | Interstate movement for <i>Phytophthora</i> screening; no outdoor release. | No significant differences in <i>Phytophthora</i> susceptibility between transgenic and non-transgenic seedlings (Section 9.2; McKeever, 2019). Initial screening in 2018 not performed due to infestations by greenhouse pests. |
| 17-053-103r | 6/15/2017 | NY | Outdoor plantings to evaluate growth, controlled pollinations, ecological interactions, and blight tolerance. | Pollination was successful with approximately expected inheritance rates. Growth was similar between transgenic and non-transgenic seedlings, with vigorous growth and high survival for all types. No deleterious effects or non-target interactions were observed. <i>C. parasitica</i> inoculations showed very good blight tolerance. |
| 17-106-101n, 16-040-102n, 15-120-106n | 5/11/2017, 2/25/2016, 5/4/2015 | NC | Interstate movement for <i>Phytophthora</i> screening. | Results inconclusive (or experiments not completed) due to high mortality of both transgenic and NT control trees (all were tissue culture-generated T0 lines). |
| 14-022-102r-a2 | 4/23/2014 | NY | Outdoor plantings to evaluate growth, controlled pollinations, and blight tolerance. | Growth of tissue culture-generated trees, including transgenic and NT controls, was slow and mortality was high. Initial pollinations successful. Preliminary screen showed good blight tolerance. |
| 10-357-118r-a1 | 3/25/2011 | NY | Growth, interactions | Legacy events (e.g. Darling 4) only |
| Authorizations owned by collaborators | | Institution | | |
| 19-168-101rm | 10/21/2019 | VA | Virginia Tech | |
| 19-092-105r | 7/29/2019 | IN | Purdue University | |
| 19-064-103rm | 7/3/2019 | PA | Pennsylvania State University | |
| 19-148-102n | 6/25/2019 | ME | University of New England | |
| 19-081-107r | 6/21/2019 | VA | The American Chestnut Foundation | |
| 19-098-102n | 5/15/2019 | VA | Virginia Tech | |
| 19-065-107n | 5/15/2019 | VA | The American Chestnut Foundation | |
| 19-056-105n | 4/1/2019 | IN | Purdue University | |
| 17-139-102r | 7/21/2017 | GA | University of Georgia | |
| 17-073-104r | 6/23/2017 | VA | The American Chestnut Foundation | |
| 17-073-103n | 5/15/2017 | NC, VA | The American Chestnut Foundation | |
| 16-180-103rm-a4 | 8/20/2016 | VA | Virginia Tech | |

Adapted and updated from Chapter 13, *Agrobacterium Protocols v2, 3rd ed.* (Maynard et al., 2015)

1. Tween 20 solution: 1% (v/v).
2. Bleach solution: 50% (v/v) unscented household bleach, two drops Tween 20 per 100 mL.
3. *Agrobacterium* growth medium: 25 g/L Luria-Bertani broth, Miller modification (Phytotechnology Laboratories, 2012), pH 7.5; add 50 mg/L kanamycin after autoclaving.
4. Virulence induction medium: 2.3 g/L Woody Plant Modified Basal Salt Medium (WPM salts) (Lloyd and McCown, 1980), 10 g/L sucrose, and 9.75 g/L 2-(N-morpholino)-ethanesulfonic acid (MES), pH 5.5; add 100 µM acetosyringone after autoclaving.
5. Embryo initiation medium (E1) (Merkle et al., 1991): 2.3 g/L WPM salts, 109 mg/L Nitsch and Nitsch vitamins (Nitsch & Nitsch, 1969), 1 g/L casein hydrolysate, 1.8 mg/L 2,4-dichlorophenoxyacetic acid, 1.1 mg/L 6-benzylaminopurine, 30 g/L sucrose, 3 g/L Phytigel, pH 5.5.
6. *Agrobacterium* kill medium (Agro Kill): E1 medium; add 50 mg/L cefotaxime and 333 mg/L timentin after autoclaving.
7. Liquid selection medium: E1 medium (without Phytigel); add 50 mg/L cefotaxime, 200 mg/L timentin, and 143 mg/L paromomycin after autoclaving.
8. Embryo initiation medium with paromomycin: E1 medium; add 143 mg/L paromomycin after autoclaving.
9. Embryo development medium (E2) (Robichaud et al., 2004): 2.3 g/L WPM salts, 1 g/L casein hydrolysate, 0.5 g/L L-glutamine, 60 g/L sucrose, 3.5 g/L Phytigel, pH 5.5.
10. Embryo maturation medium (E3) (Xing et al., 1999): 3.08 g/L Gamborg Basal Salt Mixture (B-5 salts) (Gamborg et al., 1968), 2.2 mg/L 6-benzylaminopurine, 2.6 mg/L α-naphthaleneacetic acid, 60 g/L sucrose, 3.5 g/L Phytigel, pH 5.5.
11. Embryo germination medium (E4) (Xing et al., 1999): 2.3 g/L WPM salts, 500 mg/L MES, 500 mg/L polyvinylpyrrolidone (PVP-40), 30 g/L sucrose, 3.5 g/L Phytigel, pH 5.5.
12. Pre-rooting medium (PR) (Xing et al., 1997): 2.3 g/L WPM salts, 109 mg/L Nitsch and Nitsch vitamins, 500 mg/L MES, 500 mg/L PVP-40, 1.0 mg/L 6-benzylaminopurine, 30 g/L sucrose, 3.5 g/L Phytigel, pH 5.5.

Appendix III. Sequence of p35S-OxO T-DNA and Darling 58 insertion site data

T-DNA sequence of p35S-OxO vector; i.e. new DNA used to transform Darling 58. Table shows locations of relevant genetic elements and PCR primer binding sites. (See Figures 7.1a and 10.4.1a for schematic representation and orientation of genetic elements.)

| Base Pair Location | p35S-OxO Genetic Element (see Section 7.1) |
|----------------------------------|--|
| 1 - 21 | Right Border (<i>italic</i>) |
| 204 - 970 | CaMV 35s Promoter (<u>underlined</u>) |
| 971 - 1728 | Oxalate Oxidase (highlighted green) |
| 1729 - 2498 | ACTII Terminator (bold) |
| 2499 - 3808 | UBQ10 Promoter (<u>underlined</u>) |
| 3809 - 4648 | NPTII (highlighted purple) |
| 4649 - 4869 | NOS Terminator (bold) |
| 5009 - 5095 | 2 Left Borders (<i>italic</i>) |
| Forward, Reverse Primer Location | Primer Name (purpose; see also Table 7.2.1a) |
| 4323 - 4342, 4427 - 4446 | NPTII (copy number qPCR) |
| 2085 - 2104, 1281 - 1300 | OxO (sequencing) |
| 2072 - 2095, 1904 - 1927 | IDT1 (copy number qPCR, expression RT-qPCR) |
| 762 - 781, genome* | SX58 down/forward (genome walking) |
| genome*, 4845 - 4864 | SX58 up/reverse (genome walking) |
| 1505 - 1522, 2074 - 2091 | LN (OxO detection PCR) |

*For genome walking sequences, the opposing primer is located in the chestnut genome, so is not present in this T-DNA map (see genome walking sequences below).

```

1   gatctgggaa accctgtggt tggcatgcac atacaatgg acgaacggat aaacctttc acgcctttt aaatatccga
81  ttattctaat aaacgctctt ttctcttagg tttaccggcc aatatatct gtcaaacact gatagttaa acttttaatt
161 aaggtagctg cagaagctag caagttaca gaagctgca tgccctgcaga ggtcaacatg gtggagcacg acacacttgt
241 ctactccaaa aatatcaag atacagtctc agaagaccaa agggcaattg agactttca acaaaggta atatccggaa

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Appendix III. Sequence of p35S-OxO T-DNA and Darling 58 insertion site data

321 accctcctcg attccattgc ccagctatct gtcactttat tgtgaagata gtggaaaagg aagggtggctc ctacaaatgc
401 catcattgcg ataaaggaaa ggccatcggt gaagatgct ctcggcagag tggcccggaa gatggacccc caccacagag
481 gagcatcgta gaaaaagaag acgttcaac cacgtcttca aagcaagtgg attgtatgtga taacatggta gggcacgaca
561 cacttgtcta ctccaaaaat atcaaagata cagtcctcaga agaccaaagg gcaattgaga ctttcaaca aagggtata
641 tcggaaacc tcctcggtt ccattgccc gctatctgtc actttattgtt gaagatagtgy gaaaaggaaag gtggctccata
721 caaatgccat cattgcata aaggaaaggc catcggttga gatgcctctg cccacagtg tcccaagat ggaccccccac
801 ccacgaggag catcggttga aaagaagacg ttccaaccac gtcttcaag caagtggatt gatgtatat ctccactgac
881 gtaaggatg acgcacaatc ccactatcct tcgcaagacc cttccctat ataaggaagt tcatttcatt tggagaggac
961 ctgcagaattt ccgcagcagc aacaaccatg gccatagaca ctctccatca acaaactcta gctgataat cctagctaag
1041 ctattacat agcaagcatg gggtaatcctt aaaccctagt agctggctg ttcgcaatgc tgtaactagc tccggccgtc
1121 ttggccaccg acccagaccc tctccaggac ttctgtgtc cgcacatcg cggcaaggcg gtctcggtga acgggcacac
1201 gtgcaagccc atgtcgagg cccgcacga ctcccttc tcgtccaatg tggcaaggc cggcaacacg tccaccccg
1281 acggctccgc cgtgacggag ctgcacgtgg ccgagtgccc cggtaccaac acgctgggtg tgccatgaa ccgcgtggac
1361 tttgctcccg gaggcacca cccaccacac atccaccggc gtgcaccga gatcggcatc gtatgaaag gtgagttct
1441 cgtggaaatc ctggcagcc tcgactccgg gaacaagctc tactcgaggg tggcgcgc cggagagacg ttccatcc
1521 cacggggcct catgcacttc cagttcaacg tcggttaagac cgaggctcc atggctgtc cttcaacag ccagaacccc
1601 ggcattgtct tcgtccccct cagctctt ggcttcaacc cggccatccc aacgcgggtg ctaccaagg cactccgggt
1681 ggaggccagg gtcgttgaac ttctcaagtc caagtttggc gctgggttt aatttcttagg agcctccct gaaatgataa
1761 ttatataattt ccatatatgc atgttagcaa aatttataaa ttcttacccag aagacatgtt tcaagtttcc aggttaatct
1841 cgcatgttagt cgttaataaa gatgtacaa gttagctca tggtagtgc ttcgatcaga accaatatga gaaattgaaat
1921 gtaactatcc ttattgtcg ttttttctt ttcaatgc ggaatataataa ataaatgtt tctgtacgtc tggaggccgg
2001 ccaatcatg gtaagcttc aagatcaag gctttttttt atgaatggg tcaaagtttcc tttttttctt
2081 ttatatttgc ttccatctt gttttttca tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
2161 ctgggtttttt acttacgtt gctttttttt aaaaaaaaaaaaac gctttttttt tttttttttt tttttttttt tttttttttt
2241 gagtgtatcg atttatgttcc ttttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
2321 ttgcataatgt taatataatg attgtataag attttttttt tttttttttt tttttttttt tttttttttt tttttttttt
2401 ttggactga tattctgaat agttaaagcg ttacatgttcc tccatcaca aatgaactta ggcgcgtac cctctagtca
2481 aggccttaggc ggcgcgtca cggatcaggat tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
2561 tctaggaccg gataagtcc ctcttcata gcgaacttat tttttttttt tttttttttt tttttttttt tttttttttt
2641 taatgaaaaa atattattgg tcattggact gaacacgagt gttttttttt tttttttttt tttttttttt tttttttttt
2721 atgaattaaa taacaagaat aaatcgatc accaaaccac ttggccctttt taacgagact tttttttttt tttttttttt
2801 aagtcttattt cctatgcata tcaataatca taaaaaaa tccaaataaca ctaaaaaattt aaaaatggaaatg gataatttca
2881 caatatgttta tacgataaaag aagttacttt tccaaagaaat tcaatgttta tttttttttt tttttttttt tttttttttt
2961 aaaaaaaaaaaca aaaaggaaaaa gaaataaagc acgaagaatt tttttttttt tttttttttt tttttttttt tttttttttt
3041 ggttcaatta ttgcaattt tcagctccac cgtatattttt aaaaataaaaa cgataatgtt tttttttttt tttttttttt
3121 gatcggtttaa tctcaacggc tggatctt gacgaccgtt agaaattgtt tttttttttt tttttttttt tttttttttt
3201 aaagtgggttgc cagccggcac acacgagtcc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
3281 aatttagccaa aaacaactttt gctgttaaac aacgctcaat acacgatgtt tttttttttt tttttttttt tttttttttt
3361 agctttctcg tgaccttagt gtcctcgat tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
3441 caattcagat ttcaattttt caaaatcttta aaaaacttttctt tttttttttt tttttttttt tttttttttt tttttttttt
3521 ccttattctc tcaaaatctt cgattttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
3601 cttagatcgat agacgattttt ctgggtttga tgggtttttt tttttttttt tttttttttt tttttttttt tttttttttt
3681 atttggatcgat tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
3761 gtttggatcgat tcaattttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
3841 cttccggccgc ttgggtggag aggcttattcg gctatgactg ggcacaaacag acaatcggtt gctctgttcc cggcgttcc
3921 cggctgtcag cgcaggggcg cccgggttcc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4001 agcgcggcta tcgtggctgg ccacgcggg cgggtttcc tttttttttt tttttttttt tttttttttt tttttttttt
4081 ggctgttattt gggcgaatgtt cggggggcagg atctctgttcc tttttttttt tttttttttt tttttttttt tttttttttt
4161 gatcgatcgat ggcggctcgat tacgttttgc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4241 acgtactcgat atggaaaggcc gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4321 tcggccaggctt caaggccgcg atggccgacg gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4401 gtggaaaatgg tggccctttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4481 taccctgtat atggatcgat agttggccg tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4561 cgcagcgcata cggcttcttgc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4641 gattgaatcc ttggccggat tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4721 taatgtatcgat tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4801 aatatacgatcgat tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4881 gtaaaactaaa gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
4961 aacaatatctt agaccgcggt gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
5041 agccaacacgc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt

Appendix III. Sequence of p35S-OxO T-DNA and Darling 58 insertion site data

Genome Walking data, showing DNA sequences from Darling 58 overlapping vector insert to endogenous *Castanea* genome. See Figure 7.2.3b for sequence alignments. No extraneous vector backbone or *Agrobacterium* sequences are present. Sequencing was completed by GENEWIZ (South Plainfield, NJ) on PCR products amplified at ESF from Darling 58 tissues. See Table 7.2.1a for sequencing primers.

Darling 58 Upstream, partial vector sequence including right border to flanking chestnut genome region. Underlined portion is inside T-DNA; remainder is flanking genome sequence:

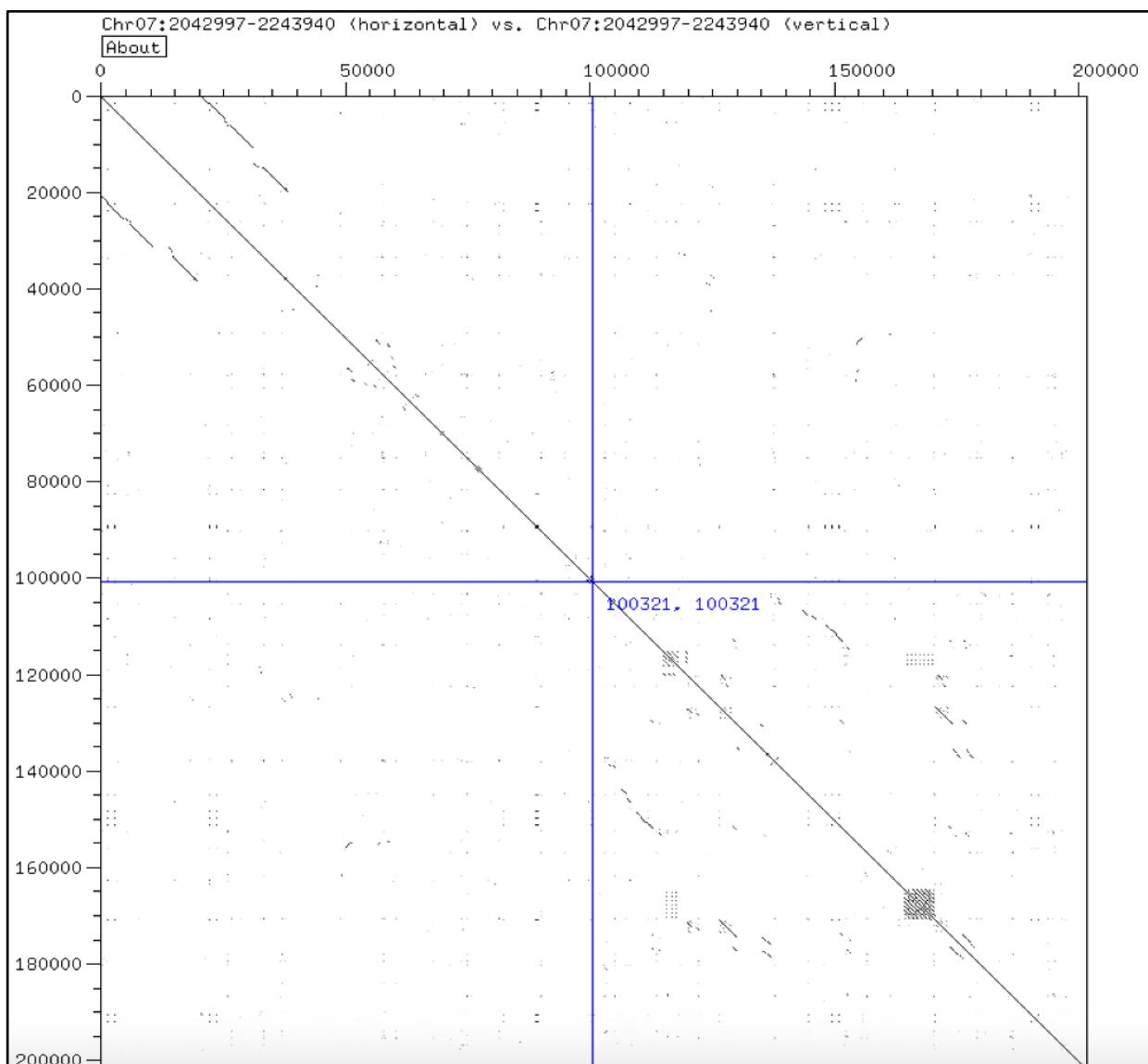
CTGACCGCTTCCTCGTGCCTACGGTATGCCGCTCCGATTGCGAGCGCATGCCCTCTATGCCCTCTTGACGAGTTCTCTGAGGGATCGTTCAAACATTGGCAATAAAGTTCTTAAGATTGAATCCTGTTGCCGGTCTGCGATGATTATCATATAATTCTGTTGAATTACGTAAAGCATGTAATAATTAAACATGTAATCTTGTTCCTTTATCAAAGGTATACATGTGTAGGTTTAATGTTTATAATCGAATGAAGAAAGTCAAATAATGTAAGAGAAAAACAAGAGCAATGGTTAATATAATTGATTCACGGTCAACCATGTCCACACCCAAAAATAAAATAAAATCAAACCTAAATTTTATGATTCTAAATTATTTGTA TGTTACAATGAAACCGTGCACGCAAGTAGACATGGCAAAACGAGTTGGGATTCAACCTGACCCAATCCAAGGAAAATAATTGATCCAATCTGATTGGTACCCGAATATAAAACAAGTTGACCTGTGACCTGACC

Darling 58 Downstream, partial vector sequence including left border to flanking chestnut genome region. (Note this sequence is a reverse complement compared to T-DNA sequence shown above). Underlined portion is inside T-DNA; remainder is flanking genome sequence:

CATAAAAGTGACAGATAGCTGGCAATGGAATCCGAGGAGGTTCCGGATATTACCTTGTGAAAAGTCTCAATTGCCCTTGGCTTCTGAGACTGTATTTGATATTTGGAGTAGACAAGTGTGTCGTGCTCCACCATGTTGACCTCTGCAGGCATGCAA GCTTCTGTTAACCTGCTAGCTTCTGCAGGTACCTTAATTAAAGTTAAATGCACTGTTAAGAGCATAACAGAACATGTGATTCA ATGGTTGAATTTCAAAATATAGATTCAATAAAATTATTAAGTACATCCAATTGGCATGAAAATTGGCATGCATATTAGAATA TATAGAACATATGATTCAACAGTTGAATTTCAAAATATAAATTCAATAACAAGTTATTGGATGATGTAACATATAATGTCAT CCAAATACAATGGC

Appendix III. Sequence of p35S-OxO T-DNA and Darling 58 insertion site data

Putative Darling 58 transgene insertion site, shown on a dot plot created from paired-end Illumina sequence data from Darling 58 genomic DNA. This plot shows the position of the T-DNA insert on a ~200,000 bp segment of Ellis Chromosome 7. Two axes represent paired reads of Darling 58 genomic DNA; diagonal line shows sequence alignment between the two reads. Dark square at the approximate position 170000, 170000 represents the T-DNA insert, preliminarily indicating its position approximately 30,000 bp from the end of Chromosome 7. (Data and preliminary analysis from Jeremy Schmutz, Hudson Alpha Institute for Biotechnology, Huntsville, AL.) Further sequence analysis of Darling 58 and transgenic offspring is underway; results will be published and/or shared when they become available.



Appendix IV. qPCR reference genes

Selecting and testing reference genes for expression studies in American chestnut. Compiled from work by Baier and Powell at ESF; previously unpublished. Subsequent pages include qPCR reports.

1. Literature was searched for examples of genes commonly used as internal reference. Genes included: GAPDH, ACTB, ACT11, eF1a, eF4a, TBP, 18s rRNA, L13a, actin, ubiquitin, and beta tubulin.
2. Fagaceae website was searched for homologous genes in American and Chinese Chestnut (AC and CC, respectively). GAPDH, eF1 α and L13a were chosen for analysis as reference genes. Actin was selected and tested later (see subsequent pages).
3. Several primer sets were designed for each gene in regions homologous to both AC and CC.
4. Optimal anneal temperature was determined for primer pairs using the temperature gradient feature of the BioRad MiniOpticon and Chinese chestnut stem tissue RNA as the template. The BioRad iScript One-Step RT-PCR kit was used. This step also indicated amplification of non-specific products or primer dimers. At this point several primer pairs were removed from consideration.
5. PCR efficiency was determined for primer pairs by using a 10-fold dilution series of CC RNA as the template (see table below). Efficiency in American chestnut template DNA was tested later (see subsequent pages in this Appendix). Desirable efficiency ranges between 90 – 105%.
6. Stability of expression was determined by comparing amplification using RNA from 2 inoculated and 2 non-inoculated stems from both chestnut species.

| Reference Gene | Primer pair # | Non-specific amplification | PCR Efficiency (%) |
|----------------|---------------|----------------------------|--------------------|
| GAPDH | 1 | No | 100.3 |
| L13a | 1 | Yes | |
| | 2 | No | 104.5 |
| EF1 α | 1 | Yes | |
| | 2 | No | 101.8 |



four primer sets 2.pcrd

09/02/2019 13:52

Report Information

User: BioRad/admin

Data File Name: four primer sets 2.pcrd

Data File Path: C:\Users\User\Desktop\dakota backup\Desktop\DFM 2018

Well Group Name: All Wells

Report Differs from Last Save: Yes

Run Setup

Run Information

Run Date: 09/30/2018 16:26

Run User: admin

Run Type: User-defined

Plate File: plate with standard for all four primer sets.pltd

ID:

Notes:

Sample Volume: 25

Temperature Control Mode: Calculated

Lid Temperature: 105

Base Serial Number: BR007351

Optical Head Serial Number: 788BR07280

Protocol

1: 95.0°C for 3:00

2: 95.0°C for 0:10

3: 60.0°C for 0:30

Plate Read

4: GOTO 2, 39 more times

5: 95.0°C for 0:10

6: Melt Curve 65.0°C to 95.0°C: Increment 0.5°C 0:05

Plate Read

Appendix IV. qPCR reference genes

Plate Display

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-------------------------------------|-------------------------------------|-------------------------------------|--------------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|---|----|----|----|
| A | Std-1 Actin Seed 001 Actin | Std-3 Actin Seed 001 Actin | Std-6 GAPDH Seed 001 GADPH | Std-9 GAPDH Seed 001 GADPH | Std-11 EF1 Seed 001 EF1 | Std-14 EF1 Seed 001 EF1 | Std-17 OxO Seed 001 OxO | Std-19 OxO Seed 001 OxO | | | | |
| B | Std-1 Actin Seed 001 Actin | Std-4 Actin Seed 001 Actin | Std-6 GAPDH Seed 001 GADPH | Std-9 GAPDH Seed 001 GADPH | Std-12 EF1 Seed 001 EF1 | Std-14 EF1 Seed 001 EF1 | Std-17 OxO Seed 001 OxO | Std-20 OxO Seed 001 OxO | | | | |
| C | Std-1 Actin Seed 001 Actin | Std-4 Actin Seed 001 Actin | Std-7 GAPDH Seed 001 GADPH | Std-9 GAPDH Seed 001 GADPH | Std-12 EF1 Seed 001 EF1 | Std-15 EF1 Seed 001 EF1 | Std-17 OxO Seed 001 OxO | Std-20 OxO Seed 001 OxO | | | | |
| D | Std-2 Actin Seed 001 Actin | Std-4 Actin Seed 001 Actin | Std-7 GAPDH Seed 001 GADPH | Std-10 GAPDH Seed 001 GADPH | Std-12 EF1 Seed 001 EF1 | Std-15 EF1 Seed 001 EF1 | Std-18 OxO Seed 001 OxO | Std-20 OxO Seed 001 OxO | | | | |

Plate Display

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-------------------------------------|-------------------------------------|-------------------------------------|--------------------------------------|----------------------------------|----------------------------------|----------------------------------|---|---|----|----|----|
| E | Std-2 Actin Seed 001 Actin | Std-5 Actin Seed 001 Actin | Std-7 GAPDH Seed 001 GADPH | Std-10 GAPDH Seed 001 GADPH | Std-13 EF1 Seed 001 EF1 | Std-15 EF1 Seed 001 EF1 | Std-18 OxO Seed 001 OxO | | | | | |
| F | Std-2 Actin Seed 001 Actin | Std-5 Actin Seed 001 Actin | Std-8 GAPDH Seed 001 GADPH | Std-10 GAPDH Seed 001 GADPH | Std-13 EF1 Seed 001 EF1 | Std-16 OxO Seed 001 OxO | Std-18 OxO Seed 001 OxO | | | | | |
| G | Std-3 Actin Seed 001 Actin | Std-5 Actin Seed 001 Actin | Std-8 GAPDH Seed 001 GADPH | Std-11 EF1 Seed 001 EF1 | Std-13 EF1 Seed 001 EF1 | Std-16 OxO Seed 001 OxO | Std-19 OxO Seed 001 OxO | | | | | |
| H | Std-3 Actin Seed 001 Actin | Std-6 GAPDH Seed 001 GADPH | Std-8 GAPDH Seed 001 GADPH | Std-11 EF1 Seed 001 EF1 | Std-14 EF1 Seed 001 EF1 | Std-16 OxO Seed 001 OxO | Std-19 OxO Seed 001 OxO | | | | | |

Quantification

Step #: 3

Analysis Mode: Fluorophore

Cq Determination: Single Threshold

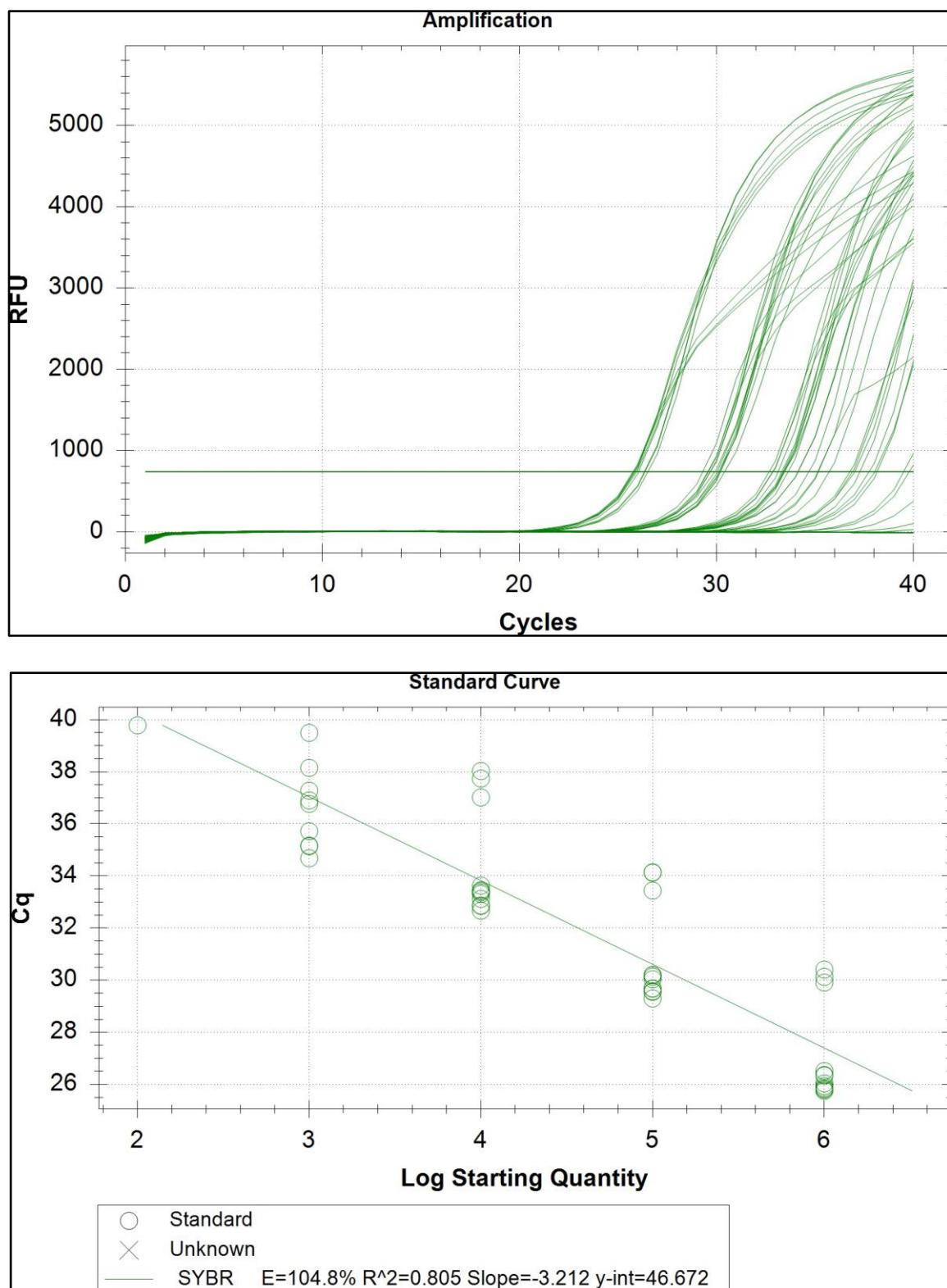
Baseline Method:

SYBR: Auto Calculated

Threshold Setting:

SYBR: 737.04, Auto Calculated

Appendix IV. qPCR reference genes



Appendix IV. qPCR reference genes

Quantification Data

| Well | Fluor | Target | Content | Sample | Biological Set Name | Cq | Cq Mean | Cq Std. Dev | Starting Quantity (SQ) | Log Starting Quantity | SQ Mean | SQ Std. Dev |
|------|-------|--------|---------|----------|---------------------|-------|---------|-------------|------------------------|-----------------------|----------|-------------|
| A01 | SYBR | Actin | Std-01 | Seed 001 | Actin | 26.04 | 25.92 | 0.115 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| A02 | SYBR | Actin | Std-03 | Seed 001 | Actin | 33.31 | 33.21 | 0.305 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| A03 | SYBR | GAPDH | Std-06 | Seed 001 | GADPH | 25.76 | 25.83 | 0.096 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| A04 | SYBR | GAPDH | Std-09 | Seed 001 | GADPH | 36.77 | 35.95 | 1.149 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 |
| A05 | SYBR | EF1 | Std-11 | Seed 001 | EF1 | 26.51 | 26.41 | 0.094 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| A06 | SYBR | EF1 | Std-14 | Seed 001 | EF1 | 37.27 | 35.71 | 1.378 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 |
| A07 | SYBR | OxO | Std-17 | Seed 001 | OxO | 34.14 | 33.90 | 0.401 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| A08 | SYBR | OxO | Std-19 | Seed 001 | OxO | 38.15 | 38.82 | 0.951 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 |
| B01 | SYBR | Actin | Std-01 | Seed 001 | Actin | 25.88 | 25.92 | 0.115 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| B02 | SYBR | Actin | Std-04 | Seed 001 | Actin | 36.90 | 36.31 | 0.837 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 |
| B03 | SYBR | GAPDH | Std-06 | Seed 001 | GADPH | 25.80 | 25.83 | 0.096 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| B04 | SYBR | GAPDH | Std-09 | Seed 001 | GADPH | N/A | 0.00 | 0.000 | 1.000E+03 | 3.000 | 0.00E+00 | 0.00E+00 |
| B05 | SYBR | EF1 | Std-12 | Seed 001 | EF1 | 30.19 | 30.18 | 0.037 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| B06 | SYBR | EF1 | Std-14 | Seed 001 | EF1 | 35.17 | 35.71 | 1.378 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 |
| B07 | SYBR | OxO | Std-17 | Seed 001 | OxO | 33.44 | 33.90 | 0.401 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| B08 | SYBR | OxO | Std-20 | Seed 001 | OxO | 39.78 | 39.78 | 0.000 | 1.000E+02 | 2.000 | 1.00E+02 | 0.00E+00 |

Appendix IV. qPCR reference genes

Quantification Data

| Well | Fluor | Target | Content | Sample | Biological Set Name | Cq | Cq Mean | Cq Std. Dev | Starting Quantity (SQ) | Log Starting Quantity | SQ Mean | SQ Std. Dev |
|------|-------|--------|---------|----------|---------------------|-------|---------|-------------|------------------------|-----------------------|----------|-------------|
| C01 | SYBR | Actin | Std-01 | Seed 001 | Actin | 25.83 | 25.92 | 0.115 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| C02 | SYBR | Actin | Std-04 | Seed 001 | Actin | N/A | 0.00 | 0.000 | 1.000E+03 | 3.000 | 0.00E+00 | 0.00E+00 |
| C03 | SYBR | GAPDH | Std-07 | Seed 001 | GADPH | 30.04 | 29.63 | 0.375 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| C04 | SYBR | GAPDH | Std-09 | Seed 001 | GADPH | 35.14 | 35.95 | 1.149 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 |
| C05 | SYBR | EF1 | Std-12 | Seed 001 | EF1 | 30.20 | 30.18 | 0.037 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| C06 | SYBR | EF1 | Std-15 | Seed 001 | EF1 | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |
| C07 | SYBR | OxO | Std-17 | Seed 001 | OxO | 34.13 | 33.90 | 0.401 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| C08 | SYBR | OxO | Std-20 | Seed 001 | OxO | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |
| D01 | SYBR | Actin | Std-02 | Seed 001 | Actin | 29.67 | 29.64 | 0.059 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| D02 | SYBR | Actin | Std-04 | Seed 001 | Actin | 35.71 | 36.31 | 0.837 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 |
| D03 | SYBR | GAPDH | Std-07 | Seed 001 | GADPH | 29.55 | 29.63 | 0.375 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| D04 | SYBR | GAPDH | Std-10 | Seed 001 | GADPH | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |
| D05 | SYBR | EF1 | Std-12 | Seed 001 | EF1 | 30.13 | 30.18 | 0.037 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| D06 | SYBR | EF1 | Std-15 | Seed 001 | EF1 | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |
| D07 | SYBR | OxO | Std-18 | Seed 001 | OxO | 38.03 | 37.59 | 0.523 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| D08 | SYBR | OxO | Std-20 | Seed 001 | OxO | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |
| E01 | SYBR | Actin | Std-02 | Seed 001 | Actin | 29.58 | 29.64 | 0.059 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| E02 | SYBR | Actin | Std-05 | Seed 001 | Actin | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |
| E03 | SYBR | GAPDH | Std-07 | Seed 001 | GADPH | 29.30 | 29.63 | 0.375 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| E04 | SYBR | GAPDH | Std-10 | Seed 001 | GADPH | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |
| E05 | SYBR | EF1 | Std-13 | Seed 001 | EF1 | 33.63 | 33.38 | 0.253 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| E06 | SYBR | EF1 | Std-15 | Seed 001 | EF1 | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |

Appendix IV. qPCR reference genes

| | | | | | | | | | | | | |
|-----|------|-------|--------|----------|-------|-------|-------|-------|-----------|-------|----------|----------|
| E07 | SYBR | OxO | Std-18 | Seed 001 | OxO | 37.74 | 37.59 | 0.523 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| F01 | SYBR | Actin | Std-02 | Seed 001 | Actin | 29.68 | 29.64 | 0.059 | 1.000E+05 | 5.000 | 1.00E+05 | 0.00E+00 |
| F02 | SYBR | Actin | Std-05 | Seed 001 | Actin | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |
| F03 | SYBR | GAPDH | Std-08 | Seed 001 | GADPH | 32.67 | 32.98 | 0.390 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| F04 | SYBR | GAPDH | Std-10 | Seed 001 | GADPH | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |
| F05 | SYBR | EF1 | Std-13 | Seed 001 | EF1 | 33.12 | 33.38 | 0.253 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |

Quantification Data

| Well | Fluor | Target | Content | Sample | Biological Set Name | Cq | Cq Mean | Cq Std. Dev | Starting Quantity (SQ) | Log Starting Quantity | SQ Mean | SQ Std. Dev |
|------|-------|--------|---------|----------|---------------------|-------|---------|-------------|------------------------|-----------------------|----------|-------------|
| F06 | SYBR | OxO | Std-16 | Seed 001 | OxO | 30.41 | 30.15 | 0.250 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| F07 | SYBR | OxO | Std-18 | Seed 001 | OxO | 37.01 | 37.59 | 0.523 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| G01 | SYBR | Actin | Std-03 | Seed 001 | Actin | 32.87 | 33.21 | 0.305 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| G02 | SYBR | Actin | Std-05 | Seed 001 | Actin | N/A | 0.00 | 0.000 | 1.000E+02 | 2.000 | 0.00E+00 | 0.00E+00 |
| G03 | SYBR | GAPDH | Std-08 | Seed 001 | GADPH | 33.42 | 32.98 | 0.390 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| G04 | SYBR | EF1 | Std-11 | Seed 001 | EF1 | 26.37 | 26.41 | 0.094 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| G05 | SYBR | EF1 | Std-13 | Seed 001 | EF1 | 33.37 | 33.38 | 0.253 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| G06 | SYBR | OxO | Std-16 | Seed 001 | OxO | 30.13 | 30.15 | 0.250 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| G07 | SYBR | OxO | Std-19 | Seed 001 | OxO | N/A | 0.00 | 0.000 | 1.000E+03 | 3.000 | 0.00E+00 | 0.00E+00 |
| H01 | SYBR | Actin | Std-03 | Seed 001 | Actin | 33.45 | 33.21 | 0.305 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| H02 | SYBR | GAPDH | Std-06 | Seed 001 | GADPH | 25.94 | 25.83 | 0.096 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| H03 | SYBR | GAPDH | Std-08 | Seed 001 | GADPH | 32.85 | 32.98 | 0.390 | 1.000E+04 | 4.000 | 1.00E+04 | 0.00E+00 |
| H04 | SYBR | EF1 | Std-11 | Seed 001 | EF1 | 26.34 | 26.41 | 0.094 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| H05 | SYBR | EF1 | Std-14 | Seed 001 | EF1 | 34.68 | 35.71 | 1.378 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 |
| H06 | SYBR | OxO | Std-16 | Seed 001 | OxO | 29.91 | 30.15 | 0.250 | 1.000E+06 | 6.000 | 1.00E+06 | 0.00E+00 |
| H07 | SYBR | OxO | Std-19 | Seed 001 | OxO | 39.49 | 38.82 | 0.951 | 1.000E+03 | 3.000 | 1.00E+03 | 0.00E+00 |

Appendix IV. qPCR reference genes

Bar Chart

Expression analysis requires that at least two wells contain sample names, targets, and valid Cqs.

Target Names

| Name | Full Name | Reference | Auto Efficiency | Efficiency |
|-------|-----------|-----------|-----------------|------------|
| Actin | Actin | True | Yes | 93.2% |
| EF1 | EF1 | False | Yes | 109.7% |
| GAPDH | GAPDH | False | Yes | 96.9% |
| OxO | OxO | False | Yes | 136.6% |

QC Parameters

Data

| Description | Value | Use | Results | Exclude Wells | All excluded wells |
|---|-------|------|--|---------------|--------------------|
| Negative control with a Cq less than | 38 | True | | False | |
| NTC with a Cq less than | 38 | True | | False | |
| NRT with a Cq less than | 38 | True | | False | |
| Positive control with a Cq greater than | 30 | True | | False | |
| Unknown without a Cq | N/A | True | | False | |
| Standard without a Cq | N/A | True | SYBR:B4, C2, C6, C8, D4, D6, D8, E2, E4, E6, F2, F4, G2, G7. | False | |
| Efficiency greater than | 110.0 | True | | | |
| Efficiency less than | 90.0 | True | | | |
| Std Curve R^2 less than | 0.980 | True | SYBR | | |
| Replicate group Cq Std Dev greater than | 0.20 | True | SYBR:A2, A4, A6, A7, A8, B2, B6, B7, C3, C4, C7, D2, D3, D7, E3, E5, E7, F3, F5, F6, F7, G1, G3, G5, G6, H1, H3, H5, H6, H7. | False | |

Appendix V. qPCR raw data

Data exported from Bio-Rad CFX Maestro software (version 1.1); subsequent pages show data from various copy number and expression experiments as noted. $\Delta\Delta Ct$ calculations and analyses shown in the body of the petition incorporate cycle values from reference genes and the gene of interest.

Cq = Quantitation Cycle (PCR cycle at which fluorescence can be detected)

Cq is also referred to as Ct (Cycle threshold) in some sources

Cq Mean = average Cq value among technical replicates

Cq Std Dev. = standard deviation of Cq value among technical replicates

Target = gene being detected (i.e. primers used in this well)

Sample = DNA template (i.e. tree type)

Appendix V. qPCR raw data

OxO copy number, including Darling 58 T1 offspring, Darling 58, and Darling 4

| Well | Target | Sample | Cq (Cycle #) | Cq Mean | Cq Std. Dev |
|---------------|--------|------------|--------------|---------|-------------|
| C03 | Actin | D58+16007 | 26.67 | 26.48 | 0.168 |
| D03 | Actin | D58+16007 | 26.37 | 26.48 | 0.168 |
| E03 | Actin | D58+16007 | 26.39 | 26.48 | 0.168 |
| F03 | Actin | D58+16020 | 25.73 | 25.53 | 0.183 |
| G03 | Actin | D58+16020 | 25.48 | 25.53 | 0.183 |
| H03 | Actin | D58+16020 | 25.38 | 25.53 | 0.183 |
| A04 | Actin | D58+16025 | 26.06 | 25.95 | 0.114 |
| B04 | Actin | D58+16025 | 25.96 | 25.95 | 0.114 |
| C04 | Actin | D58+16025 | 25.83 | 25.95 | 0.114 |
| B02 | Actin | Darling 4 | 25.10 | 25.10 | 0.052 |
| C02 | Actin | Darling 4 | 25.04 | 25.10 | 0.052 |
| D02 | Actin | Darling 4 | 25.15 | 25.10 | 0.052 |
| E02 | Actin | Darling 58 | 25.22 | 25.15 | 0.058 |
| F02 | Actin | Darling 58 | 25.11 | 25.15 | 0.058 |
| G02 | Actin | Darling 58 | 25.12 | 25.15 | 0.058 |
| A01, B01, C01 | Actin | water | 0.00 | 0.00 | 0.000 |
| A07 | EF1 | D58+16007 | 27.14 | 27.04 | 0.096 |
| B07 | EF1 | D58+16007 | 26.95 | 27.04 | 0.096 |
| C07 | EF1 | D58+16007 | 27.03 | 27.04 | 0.096 |
| D07 | EF1 | D58+16020 | 26.22 | 26.16 | 0.057 |
| E07 | EF1 | D58+16020 | 26.16 | 26.16 | 0.057 |
| F07 | EF1 | D58+16020 | 26.10 | 26.16 | 0.057 |
| A08 | EF1 | D58+16025 | 26.25 | 26.30 | 0.055 |
| G07 | EF1 | D58+16025 | 26.36 | 26.30 | 0.055 |
| H07 | EF1 | D58+16025 | 26.29 | 26.30 | 0.055 |
| A06 | EF1 | Darling 4 | 25.40 | 25.46 | 0.056 |
| B06 | EF1 | Darling 4 | 25.50 | 25.46 | 0.056 |
| H05 | EF1 | Darling 4 | 25.48 | 25.46 | 0.056 |
| C06 | EF1 | Darling 58 | 25.64 | 25.64 | 0.020 |
| D06 | EF1 | Darling 58 | 25.62 | 25.64 | 0.020 |
| E06 | EF1 | Darling 58 | 25.66 | 25.64 | 0.020 |
| A05, G04, H04 | EF1 | water | 0.00 | 0.00 | 0.000 |
| A11 | OxO | D58+16007 | 30.40 | 30.40 | 0.051 |
| G10 | OxO | D58+16007 | 30.45 | 30.40 | 0.051 |
| H10 | OxO | D58+16007 | 30.35 | 30.40 | 0.051 |
| B11 | OxO | D58+16020 | 29.43 | 29.55 | 0.101 |

Appendix V. qPCR raw data

| | | | | | |
|---------------|-----|------------|-------|-------|-------|
| C11 | OxO | D58+16020 | 29.58 | 29.55 | 0.101 |
| D11 | OxO | D58+16020 | 29.63 | 29.55 | 0.101 |
| E11 | OxO | D58+16025 | 30.00 | 29.76 | 0.334 |
| F11 | OxO | D58+16025 | 29.52 | 29.76 | 0.334 |
| G11 | OxO | D58+16025 | 0.00 | 0.00 | 0.000 |
| F09 | OxO | Darling 4 | 28.09 | 28.00 | 0.082 |
| G09 | OxO | Darling 4 | 27.93 | 28.00 | 0.082 |
| H09 | OxO | Darling 4 | 27.98 | 28.00 | 0.082 |
| A10 | OxO | Darling 58 | 28.71 | 28.69 | 0.200 |
| B10 | OxO | Darling 58 | 28.89 | 28.69 | 0.200 |
| C10 | OxO | Darling 58 | 28.49 | 28.69 | 0.200 |
| E08, F08, G08 | OxO | water | 0.00 | 0.00 | 0.000 |

nptII copy number, including Darling 58, Darling 4, and Darling 5

| Well | Target | Sample | Cq (Cycle #) | Cq Mean |
|----------|--------|------------|--------------|---------|
| A01, A02 | ef1 | water | 0.00 | 0.00 |
| D01 | ef1 | Darling 58 | 24.94 | 24.94 |
| D02 | ef1 | Darling 58 | 25.05 | 25.05 |
| B01 | ef1 | Darling4 | 25.30 | 25.30 |
| B02 | ef1 | Darling4 | 25.31 | 25.31 |
| C01 | ef1 | Darling5 | 23.37 | 23.37 |
| C02 | ef1 | Darling5 | 23.74 | 23.74 |
| A03, A04 | NPTII | water | 0.00 | 0.00 |
| D03 | NPTII | Darling 58 | 26.34 | 26.34 |
| D04 | NPTII | Darling 58 | 26.35 | 26.35 |
| B03 | NPTII | Darling4 | 25.05 | 25.05 |
| B04 | NPTII | Darling4 | 25.13 | 25.13 |
| C03 | NPTII | Darling5 | 24.50 | 24.50 |
| C04 | NPTII | Darling5 | 24.59 | 24.59 |

Appendix V. qPCR raw data

OxO expression in leaf tissue from Darling 4, Darling 58 T0, Darling 58 T1 offspring, and Darling 311

| | | Target: Actin (Reference) | | | Target: EF1 (Reference) | | | Target: OxO (Gene of Interest) | | |
|------------------|-----------|------------------------------|------------|----------------|----------------------------|------------|----------------|-----------------------------------|------------|----------------|
| Sample | Replicate | Cq | Cq Mean | Cq Std. Dev | Cq | Cq Mean | Cq Std. Dev | Cq | Cq Mean | Cq Std. Dev |
| Ellis | 1 | 19.80 | 19.83 | 0.065 | 20.26 | 20.18 | 0.073 | n/a | 0.00 | 0.000 |
| | 2 | 19.79 | 19.83 | 0.065 | 20.12 | 20.18 | 0.073 | n/a | 0.00 | 0.000 |
| | 3 | 19.91 | 19.83 | 0.065 | 20.16 | 20.18 | 0.073 | n/a | 0.00 | 0.000 |
| Darling 4 | 1 | 19.98 | 19.95 | 0.044 | 20.90 | 20.98 | 0.067 | 23.79 | 23.81 | 0.047 |
| | 2 | 19.97 | 19.95 | 0.044 | 21.02 | 20.98 | 0.067 | 23.77 | 23.81 | 0.047 |
| | 3 | 19.90 | 19.95 | 0.044 | 21.02 | 20.98 | 0.067 | 23.86 | 23.81 | 0.047 |
| Darling 58 | 1 | 21.08 | 21.08 | 0.015 | 20.99 | 21.02 | 0.025 | 16.96 | 17.10 | 0.161 |
| | 2 | 21.10 | 21.08 | 0.015 | 21.04 | 21.02 | 0.025 | 17.06 | 17.10 | 0.161 |
| | 3 | 21.07 | 21.08 | 0.015 | 21.02 | 21.02 | 0.025 | 17.27 | 17.10 | 0.161 |
| D58+16001 (T1) | 1 | 20.13 | 19.94 | 0.169 | 20.81 | 20.76 | 0.052 | 16.85 | 16.89 | 0.095 |
| | 2 | 19.81 | 19.94 | 0.169 | 20.71 | 20.76 | 0.052 | 16.82 | 16.89 | 0.095 |
| | 3 | 19.87 | 19.94 | 0.169 | 20.75 | 20.76 | 0.052 | 17.00 | 16.89 | 0.095 |
| D58+16007 (T1) | 1 | 19.92 | 19.89 | 0.037 | 20.75 | 20.69 | 0.064 | 16.60 | 16.70 | 0.123 |
| | 2 | 19.85 | 19.89 | 0.037 | 20.63 | 20.69 | 0.064 | 16.84 | 16.70 | 0.123 |
| | 3 | 19.90 | 19.89 | 0.037 | 20.68 | 20.69 | 0.064 | 16.67 | 16.70 | 0.123 |
| D58+16020 (T1) | 1 | 20.13 | 20.13 | 0.048 | 20.76 | 20.71 | 0.064 | 16.48 | 16.59 | 0.114 |
| | 2 | 20.18 | 20.13 | 0.048 | 20.73 | 20.71 | 0.064 | 16.70 | 16.59 | 0.114 |
| | 3 | 20.08 | 20.13 | 0.048 | 20.63 | 20.71 | 0.064 | 16.60 | 16.59 | 0.114 |
| D58+16025 (T1) | 1 | 20.52 | 20.48 | 0.132 | 20.66 | 20.72 | 0.077 | 17.60 | 17.50 | 0.097 |
| | 2 | 20.33 | 20.48 | 0.132 | 20.80 | 20.72 | 0.077 | 17.41 | 17.50 | 0.097 |
| | 3 | 20.59 | 20.48 | 0.132 | 20.69 | 20.72 | 0.077 | 17.47 | 17.50 | 0.097 |
| Darling 311 (T1) | 1 | 21.22 | 21.21 | 0.037 | 21.23 | 21.21 | 0.021 | 17.95 | 17.84 | 0.109 |
| | 2 | 21.25 | 21.21 | 0.037 | 21.19 | 21.21 | 0.021 | 17.73 | 17.84 | 0.109 |
| | 3 | 21.17 | 21.21 | 0.037 | 21.20 | 21.21 | 0.021 | 17.83 | 17.84 | 0.109 |
| Water | 1 | | 0.00 | 0.000 | | 0.00 | 0.000 | | 0.00 | 0.000 |
| | 2 | | 0.00 | 0.000 | | 0.00 | 0.000 | | 0.00 | 0.000 |
| | 3 | | 0.00 | 0.000 | | 0.00 | 0.000 | | 0.00 | 0.000 |

Appendix V. qPCR raw data

OxO expression in woody stem tissue from Darling 215, Darling 58 T0, Darling 58 T1, and Darling 54

| | | Target: Actin (Reference) | | | Target: OxO (Gene of Interest) | | |
|------------------------|-----------|------------------------------|---------|-------------|-----------------------------------|---------|-------------|
| Sample | Replicate | Cq | Cq Mean | Cq Std. Dev | Cq | Cq Mean | Cq Std. Dev |
| Darling 215 | 1 | 22.46 | 24.79 | 1.879 | 18.6 | 20.57 | 1.290 |
| | 2 | 23.14 | 24.79 | 1.879 | 19.67 | 20.57 | 1.290 |
| | 3 | 24.16 | 24.79 | 1.879 | 20.43 | 20.57 | 1.290 |
| | 1 | 27.12 | 24.79 | 1.879 | 21.28 | 20.57 | 1.290 |
| | 2 | 25.22 | 24.79 | 1.879 | 21.28 | 20.57 | 1.290 |
| | 3 | 26.66 | 24.79 | 1.879 | 22.17 | 20.57 | 1.290 |
| Darling 58 Rep 1 | 1 | 24.81 | 23.51 | 1.15 | 17.11 | 17.27 | 0.21 |
| | 2 | 22.65 | 23.51 | 1.15 | 17.2 | 17.27 | 0.21 |
| | 3 | 23.06 | 23.51 | 1.15 | 17.51 | 17.27 | 0.21 |
| Darling 58 Rep 2 | 1 | 23.30 | 23.95 | 1.11 | 17.61 | 17.89 | 0.43 |
| | 2 | 23.32 | 23.95 | 1.11 | 17.68 | 17.89 | 0.43 |
| | 3 | 25.24 | 23.95 | 1.11 | 18.38 | 17.89 | 0.43 |
| Darling 58 T1 (+16001) | 1 | 24.25 | 24.68 | 0.57 | 18.42 | 18.47 | 0.17 |
| | 2 | 24.46 | 24.68 | 0.57 | 18.33 | 18.47 | 0.17 |
| | 3 | 25.32 | 24.68 | 0.57 | 18.66 | 18.47 | 0.17 |
| Darling 54 | 1 | 24.62 | 25.20 | 0.75 | 19.17 | 19.29 | 0.36 |
| | 2 | 26.05 | 25.20 | 0.75 | 19.01 | 19.29 | 0.36 |
| | 3 | 24.94 | 25.20 | 0.75 | 19.69 | 19.29 | 0.36 |
| Water | 1 | | 0.00 | 0.000 | | 0.00 | 0.000 |
| | 2 | | 0.00 | 0.000 | | 0.00 | 0.000 |
| | 3 | | 0.00 | 0.000 | | 0.00 | 0.000 |

Appendix VI. Darling 58 nutrition analyses

Final Report



Medallion Labs

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1-800-245-5615 info@medlabs.com

Completion Date: December 13, 2017

Date Submitted: November 29, 2017

Medallion Company ID: SUNY-ESF01

Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Library Number: 2017-MED-14701

PO Number: CF18-35

Email: [REDACTED]

Fax:

Medallion Labs Sample ID: 2017-MED-14701-01

Chestnuts, Raw, Peeled & Chopped

Customer Sample ID: T1 Chestnuts

| Assay Group | Test | Results | Test Date |
|--|-----------------------------|-----------------------|-----------|
| Sample Handling Processing Level 1 | Sample Process Fee | Sample Processed | 11/29/17 |
| ² Carbohydrates | Carbohydrates | 36.8 % | 12/13/17 |
| ² Calories | Calories | 207 Calories/100 g | 12/13/17 |
| | Calories from Fat | 49 Calories/100 g | |
| | Calories from Saturated Fat | 7 Calories/100 g | |
| Metals Screen 1 | Metals Screen | Discount | 11/29/17 |
| Ash, Overnight | Ash | 0.680 % | 12/06/17 |
| Moisture by Vacuum Oven (100° C/5 hrs) | Moisture | 54.526 % | 12/06/17 |
| Fats, by Gas Chromatography | Total Fat | 5.43 % | 12/07/17 |
| | Saturated Fat | 0.80 % | |
| | Monounsaturated Fat | 3.37 % | |
| | cis-cis Polyunsaturated Fat | 1.02 % | |
| | trans Fat | 0.01 % | |
| Cholesterol | Total Cholesterol | Less than 1.0 mg/100g | 12/07/17 |
| Protein, by Durnas | Protein (5.30) | 2.61 % | 12/13/17 |
| Calcium | Calcium | 46.0 mg/100g | 12/07/17 |
| Iron | Iron | <1.00 mg/100g | 12/07/17 |
| Sodium | Sodium | 6.68 mg/100g | 12/07/17 |
| Potassium | Potassium | 269 mg/100g | 12/07/17 |
| Dietary Fiber | Total Dietary Fiber | 6.1 % | 12/11/17 |
| Vitamin C | Vitamin C | <0.500 mg/100g | 12/13/17 |

Medallion Labs maintains A2LA accreditation to ISO/IEC 17025 for the specific tests listed in A2LA Certificate # 2769.01.

Medallion's services, including this report, are provided subject to all provisions of Medallion's Standard Terms and Conditions, a copy of which appears at www.medlabs.com.

Unless otherwise noted above, samples were received in acceptable condition and analyzed as received.

Limits of Detection and Measurement Variability are available upon request.

² This test is not considered in-scope of our current A2LA accreditation. For a listing of in-scope tests, please visit www.medlabs.com.

Date Issued: December 13, 2017

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Appendix VI. Darling 58 nutrition analyses

Final Report



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Completion Date: December 13, 2017

Date Submitted: November 29, 2017

Medallion Company ID: SUNY-ESF01

Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Library Number: 2017-MED-14701

PO Number: CF18-35

Email: [REDACTED]

Fax:

Medallion Labs Sample ID: 2017-MED-14701-02

Chestnuts, Raw, Peeled & Chopped

Customer Sample ID: NT Chestnuts

| Assay Group | Test | Results | Test Date |
|--|-----------------------------|-----------------------|-----------|
| Sample Handling Processing Level 1 | Sample Process Fee | Sample Processed | 11/29/17 |
| ² Carbohydrates | Carbohydrates | 36.3 % | 12/13/17 |
| ² Calories | Calories | 205 Calories/100 g | 12/13/17 |
| | Calories from Fat | 50 Calories/100 g | |
| | Calories from Saturated Fat | 7 Calories/100 g | |
| Metals Screen 1 | Metals Screen | Discount | 11/29/17 |
| Ash, Overnight | Ash | 0.631 % | 12/06/17 |
| Moisture by Vacuum Oven (100° C/5 hrs) | Moisture | 54.917 % | 12/06/17 |
| Fats, by Gas Chromatography | Total Fat | 5.52 % | 12/07/17 |
| | Saturated Fat | 0.81 % | |
| | Monounsaturated Fat | 3.43 % | |
| | cis-cis Polyunsaturated Fat | 1.03 % | |
| | trans Fat | 0.01 % | |
| Cholesterol | Total Cholesterol | Less than 1.0 mg/100g | 12/07/17 |
| Protein, by Dumas | Protein (5.30) | 2.63 % | 12/13/17 |
| Calcium | Calcium | 43.0 mg/100g | 12/07/17 |
| Iron | Iron | <1.00 mg/100g | 12/07/17 |
| Sodium | Sodium | 6.09 mg/100g | 12/07/17 |
| Potassium | Potassium | 242 mg/100g | 12/07/17 |
| Dietary Fiber | Total Dietary Fiber | 6.1 % | 12/11/17 |
| Vitamin C | Vitamin C | <0.500 mg/100g | 12/13/17 |

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Appendix VI. Darling 58 nutrition analyses

Final Report



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Completion Date: December 13, 2017

Date Submitted: November 29, 2017

Medallion Company ID: SUNY-ESF01

Company Code: 16311

Results Approved By: Kelsey Johnston

(Authorized Reviewer)

Method References:

| Assay Group | Method Reference |
|--|---------------------------------------|
| Ash, Overnight | AOAC: 923.03 |
| Calcium | AOAC: 975.03, 985.01, 984.27, 2011.14 |
| Calories | Determined through calculation |
| Carbohydrates | Determined through calculation |
| Cholesterol | AOAC: 976.26* |
| Dietary Fiber | AOAC: 991.43* |
| Fats, by Gas Chromatography | AOAC: 996.06* |
| Iron | AOAC: 975.03, 985.01, 984.27, 2011.14 |
| Moisture by Vacuum Oven (100° C/5 hrs) | AOAC: 926.08, 927.05 |
| Potassium | AOAC: 975.03, 985.01, 984.27, 2011.14 |
| Protein, by Dumas | AOAC: 992.15; AACC: 46-30 |
| Sodium | AOAC: 975.03, 985.01, 984.27, 2011.14 |
| Vitamin C | AOAC: 967.22, 984.26* |

* This method has been modified.

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Date Issued: December 13, 2017

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Appendix VI. Darling 58 nutrition analyses

Subject: Regarding Vitamin C and Iron Results on 2017-MED-14701 (Chestnut Samples)

From: Info@medlabs.com

To: [REDACTED]

Cc: [REDACTED]

Date: Tuesday, December 19, 2017, 10:15:47 AM EST

Hi,

I had each lab re-review their results, looking at the chromatography for the Vitamin C results and double checking the calculations accounted for the right dilutions for the Iron testing. Both labs reported that they are confident in the results they observed, and everything proceeded through each method as expected.

Please let me know if there is anything else I can assist you with.

Regards,

Anil

[Anil Harbaran | Medallion Labs](#)

Medallion Customer Service Consultant

P: (763)764-4453

9000 Plymouth Avenue North, Minneapolis, MN 55427

info@medlabs.com

www.medallionlabs.com

Please note, our offices will be closed, Monday, December 25th and Monday, January 1st. The last day to accept RUSH service is December 18th.



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Appendix VI. Darling 58 nutrition analyses



Medallion Labs

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Final Report

Order Number: 2018-008143

Completed Date: 05-Dec-2018

Submitted Date: 01-Nov-2018

Submitter: Andrew Newhouse

Company: SUNY-ESF

Company Address: 1 Forestry Drive
Syracuse, NY 13210

Results Email: [REDACTED]

Invoice Email: [REDACTED]

Payment Type: PO Number

Purchase Order: CF-19-34

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Date Issued: December 05, 2018

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Report #: 9601

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Appendix VI. Darling 58 nutrition analyses



Medallion Labs

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Final Report

| | | | |
|----------------------------|----------------------------------|-----------------|---|
| Order # Sample ID: | 2018-008143-04 | Company: | SUNY-ESF |
| Customer Sample ID: | Clark NT | | State University of New York College of F |
| Sample Description: | Chestnuts, Raw, Peeled & Chopped | | |

Analytical Testing

| <u>Method:</u> | <u>Component:</u> | <u>Result:</u> | <u>Test Date:</u> |
|----------------------------|---------------------------------------|--------------------|-------------------|
| Ash | Ash | 1.270 % | 06-Nov-2018 |
| ² Calories | Calories | 222 Calories/100 g | 09-Nov-2018 |
| | Calories, 2020 | 222 Calories/100 g | 09-Nov-2018 |
| | Calories from Fat | 38 Calories/100 g | 09-Nov-2018 |
| | Calories from Saturated Fat | 5 Calories/100 g | 09-Nov-2018 |
| | Calories (Insoluble Fiber Subtracted) | 222 Calories/100 g | 09-Nov-2018 |
| ² Carbohydrates | Carbohydrates | 40.8 % | 09-Nov-2018 |
| | Carbohydrates, 2020 | 40.8 % | 09-Nov-2018 |
| | Carbohydrates, Available | 40.8 % | 09-Nov-2018 |
| Fat (Gas Chromatography) | Total Fat | 4.23 % | 08-Nov-2018 |
| | Saturated Fat | 0.58 % | 08-Nov-2018 |
| | Monounsaturated Fat | 2.70 % | 08-Nov-2018 |
| | cis-cis Polyunsaturated Fat | 0.76 % | 08-Nov-2018 |
| | trans Fat | <LOQ % | 08-Nov-2018 |
| Fiber (AOAC 991.43) | Total Dietary Fiber | 7.2 % | 06-Nov-2018 |
| Metals (ICP-OES) | Calcium | 71.1 mg/100g | 07-Nov-2018 |
| Metals (ICP-OES) | Iron | <1.00 mg/100g | 07-Nov-2018 |
| Metals (ICP-OES) | Potassium | 549 mg/100g | 07-Nov-2018 |
| Metals (ICP-OES) | Sodium | <3.00 mg/100g | 07-Nov-2018 |
| Moisture by Vacuum Oven | Moisture | 48.621 % | 07-Nov-2018 |
| Protein | Protein (5.30) | 5.08 % | 08-Nov-2018 |
| Vitamin C | Vitamin C | 36.00 mg/100g | 02-Nov-2018 |

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Appendix VI. Darling 58 nutrition analyses



Medallion Labs

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Final Report

| | | | |
|----------------------------|----------------------------------|-----------------|---|
| Order # Sample ID: | 2018-008143-05 | Company: | SUNY-ESF |
| Customer Sample ID: | NT Chestnuts (T2-WT) | | State University of New York College of F |
| Sample Description: | Chestnuts, Raw, Peeled & Chopped | | |

Analytical Testing

| <u>Method:</u> | <u>Component:</u> | <u>Result:</u> | <u>Test Date:</u> |
|----------------------------|---------------------------------------|--------------------|-------------------|
| Ash | Ash | 1.223 % | 06-Nov-2018 |
| ² Calories | Calories | 240 Calories/100 g | 09-Nov-2018 |
| | Calories, 2020 | 240 Calories/100 g | 09-Nov-2018 |
| | Calories from Fat | 62 Calories/100 g | 09-Nov-2018 |
| | Calories from Saturated Fat | 9 Calories/100 g | 09-Nov-2018 |
| | Calories (Insoluble Fiber Subtracted) | 240 Calories/100 g | 09-Nov-2018 |
| ² Carbohydrates | Carbohydrates | 39.6 % | 09-Nov-2018 |
| | Carbohydrates, 2020 | 39.6 % | 09-Nov-2018 |
| | Carbohydrates, Available | 39.6 % | 09-Nov-2018 |
| Fat (Gas Chromatography) | Total Fat | 6.91 % | 08-Nov-2018 |
| | Saturated Fat | 1.01 % | 08-Nov-2018 |
| | Monounsaturated Fat | 4.43 % | 08-Nov-2018 |
| | cis-cis Polyunsaturated Fat | 1.18 % | 08-Nov-2018 |
| | trans Fat | <LOQ % | 08-Nov-2018 |
| Fiber (AOAC 991.43) | Total Dietary Fiber | 9.0 % | 06-Nov-2018 |
| Metals (ICP-OES) | Calcium | 50.0 mg/100g | 07-Nov-2018 |
| Metals (ICP-OES) | Iron | 1.06 mg/100g | 07-Nov-2018 |
| Metals (ICP-OES) | Potassium | 548 mg/100g | 07-Nov-2018 |
| Metals (ICP-OES) | Sodium | <3.00 mg/100g | 07-Nov-2018 |
| Moisture by Vacuum Oven | Moisture | 47.316 % | 07-Nov-2018 |
| Protein | Protein (5.30) | 4.91 % | 08-Nov-2018 |
| Vitamin C | Vitamin C | 51.60 mg/100g | 02-Nov-2018 |

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Appendix VI. Darling 58 nutrition analyses



Medallion Labs

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Final Report

| | | | |
|----------------------------|----------------------------------|-----------------|---|
| Order # Sample ID: | 2018-008143-06 | Company: | SUNY-ESF |
| Customer Sample ID: | T2+Chestnuts (T2 D58+) | | State University of New York College of F |
| Sample Description: | Chestnuts, Raw, Peeled & Chopped | | |

Analytical Testing

| <u>Method:</u> | <u>Component:</u> | <u>Result:</u> | <u>Test Date:</u> |
|----------------------------|---------------------------------------|--------------------|-------------------|
| Ash | Ash | 1.273 % | 06-Nov-2018 |
| ² Calories | Calories | 234 Calories/100 g | 09-Nov-2018 |
| | Calories, 2020 | 234 Calories/100 g | 09-Nov-2018 |
| | Calories from Fat | 47 Calories/100 g | 09-Nov-2018 |
| | Calories from Saturated Fat | 7 Calories/100 g | 09-Nov-2018 |
| | Calories (Insoluble Fiber Subtracted) | 234 Calories/100 g | 09-Nov-2018 |
| ² Carbohydrates | Carbohydrates | 42.0 % | 09-Nov-2018 |
| | Carbohydrates, 2020 | 42.0 % | 09-Nov-2018 |
| | Carbohydrates, Available | 42.0 % | 09-Nov-2018 |
| Fat (Gas Chromatography) | Total Fat | 5.25 % | 08-Nov-2018 |
| | Saturated Fat | 0.77 % | 08-Nov-2018 |
| | Monounsaturated Fat | 3.37 % | 08-Nov-2018 |
| | cis-cis Polyunsaturated Fat | 0.89 % | 08-Nov-2018 |
| | trans Fat | <LOQ % | 08-Nov-2018 |
| Fiber (AOAC 991.43) | Total Dietary Fiber | 8.7 % | 06-Nov-2018 |
| Metals (ICP-OES) | Calcium | 42.5 mg/100g | 07-Nov-2018 |
| Metals (ICP-OES) | Iron | 1.07 mg/100g | 07-Nov-2018 |
| Metals (ICP-OES) | Potassium | 576 mg/100g | 07-Nov-2018 |
| Metals (ICP-OES) | Sodium | <3.00 mg/100g | 07-Nov-2018 |
| Moisture by Vacuum Oven | Moisture | 46.754 % | 07-Nov-2018 |
| Protein | Protein (5.30) | 4.73 % | 08-Nov-2018 |
| Vitamin C | Vitamin C | 53.00 mg/100g | 02-Nov-2018 |

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² This test is not considered in-scope of our current A2LA accreditation. For a listing of in-scope tests, please visit www.medallionlabs.com.

Date Issued: December 05, 2018 Medallion Labs 9000 Plymouth Ave. N., Minneapolis, MN 55427 Report #: 9601 Page 4 of 6

Appendix VI. Darling 58 nutrition analyses



Medallion Labs

www.medallionlabs.com 800-245-5615 info@medlabs.com

Final Report

Results Approved By:

Kelsey Johnston

(Authorized Reviewer)

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Report #: 9601

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Medallion Labs

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Final Report

Analytical Method References:

Method Name

Ash

Calories

Carbohydrates

Fat (Gas Chromatography)

Fiber (AOAC 991.43)

Metals (ICP-OES)

Moisture by Vacuum Oven

Protein

Vitamin C

Method Reference

AOAC: 923.03*

Please contact for Method Details

Please contact for Method Details

AOAC: 996.06*

AOAC: 991.43*

AOAC: 975.03*, 985.01*, 984.27*, 2011.14*

AOAC: 926.08, 927.05

AACC 46-30*; AOAC 992.15*

AOAC 967.22*, 984.26*

* This method has been modified.

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² This test is not considered in-scope of our current A2LA accreditation. For a listing of in-scope tests, please visit www.medallionlabs.com.

Date Issued: December 05, 2018

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Report #: 9601

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Appendix VI. Darling 58 nutrition analyses



Fatty Acid Profile

Medallion Labs

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Sample ID: 2018-008143-04
Description: Chestnuts, Raw, Peeled & Chopped
Analysis: Triglycerides

Identifier: Clark NT
Date Run: 08-Nov-2018

| Component Name | Normalized by Weight | % (w/w) as Triglyceride in Product | % (w/w) Fatty Acids in Product | | | |
|---|----------------------|------------------------------------|--------------------------------|-----------------------------|-------------------------------------|-------------------------------|
| | | | Saturated Fatty Acids | Monounsaturated Fatty Acids | cis-cis Polyunsaturated Fatty Acids | trans Unsaturated Fatty Acids |
| C-4:0 Butyric | | | | | | |
| C-6:0 Caproic | | | | | | |
| C-8:0 Caprylic | | | | | | |
| C-10:0 Capric | | | | | | |
| C-12:0 Lauric | | | | | | |
| C-13:0 Tridecanoic | | | | | | |
| C-14:0 Myristic | | | | | | |
| C-14:1 t-Tetradecanoic | | | | | | |
| C-14:1 Myristoleic | | | | | | |
| C-15:0 Pentadecanoic | | | | | | |
| C-15:1 Pentadecenoic | | | | | | |
| C-16:0 Palmitic | 11.926% | 0.504 | 0.480 | | | |
| C-16:1 t-Hexadecenoic | | | | | | |
| C-16:1 Palmitoleic | | | | | | |
| C-17:0 Margaric | | | | | | |
| C-17:1 Margaroleic | | | | | | |
| C-18:0 Stearic | 1.731% | 0.073 | 0.070 | | | |
| C-18:1 t-Elaidic | | | | | | |
| C-18:1 Oleic | 65.809% | 2.780 | 2.660 | | | |
| C-18:2 t-Octadecadienoic | | | | | | |
| C-18:2 Linoleic | 17.076% | 0.721 | 0.690 | | | |
| C-20:0 Arachidic | 0.493% | 0.021 | 0.020 | | | |
| C-18:3 g-Linolenic | | | | | | |
| C-20:1 Gadoleic | 0.986% | 0.042 | 0.040 | | | |
| C-18:3 Linolenic | 1.733% | 0.073 | 0.070 | | | |
| C-21:0 Heneicosanoic | | | | | | |
| C-18:2 conj-Linoleic | | | | | | |
| C-18:4 Octadecatetraenoic | | | | | | |
| C-20:2 Eicosadienoic | | | | | | |
| C-22:0 Behenic | 0.246% | 0.010 | 0.010 | | | |
| C-20:3 g-Eicosatrienoic | | | | | | |
| C-22:1 Erucic | | | | | | |
| C-20:3 Eicosatrienoic | | | | | | |
| C-20:4 Arachidonicoic | | | | | | |
| C-23:0 Tricosanoic | | | | | | |
| C-22:2 Docosadienoic | | | | | | |
| C-24:0 Lignoceric | | | | | | |
| C-20:5 Eicosapentaenoic | | | | | | |
| C-24:1 Nervonic | | | | | | |
| C-22:3 Docosatrienoic | | | | | | |
| C-22:4 Docosatetraenoic | | | | | | |
| C-22:5 Docosapentaenoic | | | | | | |
| C-22:6 Docosahexaenoic | | | | | | |
| C-18:3 t-Linolenic | | | | | | |
| Totals: | 100.00% | 4.22 | 0.58 | 2.70 | 0.76 | 0.00 |
| Percent of Fatty Acid Components based on Total Fat: | | | 14.36% | 66.83% | 18.81% | 0.00% |
| Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA): | | | | | 0.00% | |

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Limits of Detection, Method References and Measurement Variability are available upon request.

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Appendix VI. Darling 58 nutrition analyses



Fatty Acid Profile

Medallion Labs

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1-800-245-5615 info@medlabs.com

| Sample ID: | 2018-008143-05 | Identifier: | NT Chestnuts (T2-WT) | | | |
|---|----------------------|------------------------------------|-----------------------|-----------------------------|-------------------------------------|-------------------------------|
| | | | Date Run: | 08-Nov-2018 | % (w/w) Fatty Acids in Product | |
| Component Name | Normalized by Weight | % (w/w) as Triglyceride in Product | Saturated Fatty Acids | Monounsaturated Fatty Acids | cis-cis Polyunsaturated Fatty Acids | trans Unsaturated Fatty Acids |
| C-4:0 Butyric | | | | | | |
| C-6:0 Caproic | | | | | | |
| C-8:0 Caprylic | | | | | | |
| C-10:0 Capric | | | | | | |
| C-12:0 Lauric | | | | | | |
| C-13:0 Tridecanoic | | | | | | |
| C-14:0 Myristic | | | | | | |
| C-14:1 t-Tetradecanoic | | | | | | |
| C-14:1 Myristoleic | | | | | | |
| C-15:0 Pentadecanoic | | | | | | |
| C-15:1 Pentadecenoic | | | | | | |
| C-16:0 Palmitic | 11.390% | 0.787 | 0.750 | | | |
| C-16:1 t-Hexadecenoic | | | | | | |
| C-16:1 Palmitoleic | | | | | | |
| C-17:0 Margaric | | | | | | |
| C-17:1 Margaroleic | | | | | | |
| C-18:0 Stearic | 3.023% | 0.209 | 0.200 | | | |
| C-18:1 t-Elaidic | | | | | | |
| C-18:1 Oleic | 66.232% | 4.577 | | 4.380 | | |
| C-18:2 t-Octadecadienoic | | | | | | |
| C-18:2 Linoleic | 16.185% | 1.118 | | | 1.070 | |
| C-20:0 Arachidic | 0.753% | 0.052 | 0.050 | | | |
| C-18:3 g-Linolenic | | | | | | |
| C-20:1 Gadoleic | 0.753% | 0.052 | | 0.050 | | |
| C-18:3 Linolenic | 1.664% | 0.115 | | | 0.110 | |
| C-21:0 Heneicosanoic | | | | | | |
| C-18:2 conj-Linoleic | | | | | | |
| C-18:4 Octadecatetraenoic | | | | | | |
| C-20:2 Eicosadienoic | | | | | | |
| C-22:0 Behenic | | | | | | |
| C-20:3 g-Eicosatrienoic | | | | | | |
| C-22:1 Erucic | | | | | | |
| C-20:3 Eicosatrienoic | | | | | | |
| C-20:4 Arachidonic | | | | | | |
| C-23:0 Tricosanoic | | | | | | |
| C-22:2 Docosadienoic | | | | | | |
| C-24:0 Lignoceric | | | | | | |
| C-20:5 Eicosapentaenoic | | | | | | |
| C-24:1 Nervonic | | | | | | |
| C-22:3 Docosatrienoic | | | | | | |
| C-22:4 Docosatetraenoic | | | | | | |
| C-22:5 Docosapentaenoic | | | | | | |
| C-22:6 Docosahexaenoic | | | | | | |
| C-18:3 t-Linolenic | | | | | | |
| Totals: | 100.00% | 6.91 | 1.00 | 4.43 | 1.18 | 0.00 |
| Percent of Fatty Acid Components based on Total Fat: | | | 15.13% | 67.02% | 17.85% | 0.00% |
| Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA): | | | | | | 0.00% |

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Appendix VI. Darling 58 nutrition analyses

| Fatty Acid Profile | | | | | | |
|---|---|------------------------------------|-----------------------|-----------------------------|-------------------------------------|-------------------------------|
| Medallion Labs | | | | | | |
| Component Name | Normalized by Weight | % (w/w) as Triglyceride in Product | Saturated Fatty Acids | Monounsaturated Fatty Acids | cis-cis Polyunsaturated Fatty Acids | trans Unsaturated Fatty Acids |
| C-4:0 Butyric | | | | | | |
| C-6:0 Caproic | | | | | | |
| C-8:0 Caprylic | | | | | | |
| C-10:0 Capric | | | | | | |
| C-12:0 Lauric | | | | | | |
| C-13:0 Tridecanoic | | | | | | |
| C-14:0 Myristic | | | | | | |
| C-14:1 t-Tetradecanoic | | | | | | |
| C-14:1 Myristoleic | | | | | | |
| C-15:0 Pentadecanoic | | | | | | |
| C-15:1 Pentadecenoic | | | | | | |
| C-16:0 Palmitic | 11.176% | 0.588 | 0.560 | | | |
| C-16:1 t-Hexadecenoic | | | | | | |
| C-16:1 Palmitoleic | | | | | | |
| C-17:0 Marganic | | | | | | |
| C-17:1 Margaroleic | | | | | | |
| C-18:0 Stearic | 2.980% | 0.157 | 0.150 | | | |
| C-18:1 t-Elaidic | | | | | | |
| C-18:1 Oleic | 66.174% | 3.480 | | 3.330 | | |
| C-18:2 t-Octadecadienoic | | | | | | |
| C-18:2 Linoleic | 16.101% | 0.847 | | | 0.810 | |
| C-20:0 Arachidic | 0.792% | 0.042 | 0.040 | | | |
| C-18:3 g-Linolenic | | | | | | |
| C-20:1 Gadoleic | 0.792% | 0.042 | | 0.040 | | |
| C-18:3 Linolenic | 1.591% | 0.084 | | | 0.080 | |
| C-21:0 Heneicosanoic | | | | | | |
| C-18:2 conj-Linoleic | | | | | | |
| C-18:4 Octadecatetraenoic | | | | | | |
| C-20:2 Eicosadienoic | | | | | | |
| C-22:0 Behenic | 0.395% | 0.021 | 0.020 | | | |
| C-20:3 g-Eicosatrienoic | | | | | | |
| C-22:1 Erucic | | | | | | |
| C-20:3 Eicosatrienoic | | | | | | |
| C-20:4 Arachidonic | | | | | | |
| C-23:0 Tricosanoic | | | | | | |
| C-22:2 Docosadienoic | | | | | | |
| C-24:0 Lignoceric | | | | | | |
| C-20:5 Eicosapentaenoic | | | | | | |
| C-24:1 Nervonic | | | | | | |
| C-22:3 Docosatrienoic | | | | | | |
| C-22:4 Docosatetraenoic | | | | | | |
| C-22:5 Docosapentaenoic | | | | | | |
| C-22:6 Docosahexaenoic | | | | | | |
| C-18:3 t-Linolenic | | | | | | |
| Totals: | 100.00% | 5.26 | 0.77 | 3.37 | 0.89 | 0.00 |
| Percent of Fatty Acid Components based on Total Fat: | | | 15.31% | 67.00% | 17.69% | 0.00% |
| Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA): | | | | | 0.00% | |
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Appendix VII. Darling 4 nutrition analyses

Final Report



Medallion Labs

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Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Library Number: 2016-MED-0176
PO Number: R994748

Email: [REDACTED]

Fax:

| Medallion Labs Sample ID: 2016-MED-0176-01 | | Customer Sample ID: Darling 4+ | |
|--|---------------------------------------|--------------------------------|-----------|
| Assay Group | Test | Results | Test Date |
| Sample Handling Processing Level 1 | Sample Process Fee | Sample Processed | 01/06/16 |
| ² Calories | Calories | 247 Calories/100 g | 01/25/16 |
| | Calories from Fat | 85 Calories/100 g | |
| | Calories from Saturated Fat | 12 Calories/100 g | |
| | Calories (Insoluble Fiber Subtracted) | 214 Calories/100 g | |
| ² Carbohydrates | Carbohydrates | 37.6 % | 01/25/16 |
| | Carbohydrates, Available | 29.4 % | |
| Metals Screen 1 | Metals Screen | Discount | 01/06/16 |
| Moisture by Vacuum Oven (100° C/5 hrs) | Moisture | 48.835 % | 01/12/16 |
| Ash, Overnight | Ash | 1.331 % | 01/15/16 |
| Protein, by Dumas | Protein (5.30) | 2.78 % | 01/19/16 |
| Fats, by Gas Chromatography | Total Fat | 9.45 % | 01/25/16 |
| | Saturated Fat | 1.30 % | |
| | Monounsaturated Fat | 6.03 % | |
| | cis-cis Polyunsaturated Fat | 1.69 % | |
| | trans Fat | 0.01 % | |
| Dietary Fiber | Insoluble Fiber | 8.2 % | 01/15/16 |
| | Soluble Fiber | 1.4 % | |
| | Total Fiber | 9.6 % | |
| Vitamin A Retinol | Vitamin A | Less than 50 IU/100 g | 01/17/16 |
| ² Organic Acids | Oxalic Acid | Results are less than 0.01 % | 02/01/16 |
| | Citric Acid | 0.36 % | |
| | Tartaric Acid | Results are less than 0.01 % | |
| | Malic Acid | 0.19 % | |
| | Quinic Acid | 0.28 % | |
| | Succinic Acid | Results are less than 0.01 % | |
| | Lactic Acid | Results are less than 0.01 % | |
| | Glutaric Acid | Results are less than 0.01 % | |
| | Acetic Acid | Results are less than 0.01 % | |

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Limits of Detection and Measurement Variability are available upon request.

² This test is not considered in-scope of our current A2LA accreditation. For a listing of in-scope tests, please visit www.medlabs.com.

Appendix VII. Darling 4 nutrition analyses

Final Report

 **Medallion Labs**

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Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Email: [REDACTED] Fax: [REDACTED]

Medallion Labs Sample ID: 2016-MED-0176-01 Customer Sample ID: Darling 4+

| Assay Group | Test | Results | Test Date |
|-------------|--------------|---------------|-----------|
| Calcium | Fumaric Acid | 0.02 % | |
| | Calcium | 29.6 mg/100g | 01/19/16 |
| Iron | Iron | 1.51 mg/100g | 01/19/16 |
| Sodium | Sodium | <3.00 mg/100g | 01/19/16 |
| Vitamin C | Vitamin C | 28.9 mg/100g | 01/12/16 |

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Appendix VII. Darling 4 nutrition analyses

Final Report



Medallion Labs

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Completion Date: February 02, 2016

Date Submitted: January 06, 2016

Medallion Company ID: SUNY-ESF01

Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Library Number: 2016-MED-0176

PO Number: R994748

Email: [REDACTED]

Fax:

Medallion Labs Sample ID: 2016-MED-0176-02

Chestnuts, Peeled And Cut, Frozen

Customer Sample ID: Darling 4 Minus

| Assay Group | Test | Results | Test Date |
|--|---------------------------------------|------------------------------|-----------|
| Sample Handling Processing Level 1 | Sample Process Fee | Sample Processed | 01/06/16 |
| ² Calories | Calories | 241 Calories/100 g | 01/25/16 |
| | Calories from Fat | 78 Calories/100 g | |
| | Calories from Saturated Fat | 11 Calories/100 g | |
| | Calories (Insoluble Fiber Subtracted) | 208 Calories/100 g | |
| ² Carbohydrates | Carbohydrates | 37.4 % | 01/25/16 |
| | Carbohydrates, Available | 29.3 % | |
| Metals Screen 1 | Metals Screen | Discount | 01/06/16 |
| Moisture by Vacuum Oven (100° C/5 hrs) | Moisture | 49.474 % | 01/12/16 |
| Ash, Overnight | Ash | 1.292 % | 01/15/16 |
| Protein, by Dumas | Protein (5.30) | 3.15 % | 01/19/16 |
| Fats, by Gas Chromatography | Total Fat | 8.72 % | 01/25/16 |
| | Saturated Fat | 1.20 % | |
| | Monounsaturated Fat | 5.60 % | |
| | cis-cis Polyunsaturated Fat | 1.53 % | |
| | trans Fat | 0.01 % | |
| Dietary Fiber | Insoluble Fiber | 8.1 % | 01/15/16 |
| | Soluble Fiber | 1.4 % | |
| | Total Fiber | 9.5 % | |
| Vitamin A Retinol | Vitamin A | Less than 50 IU/100 g | 01/17/16 |
| ² Organic Acids | Oxalic Acid | Results are less than 0.01 % | 01/19/16 |
| | Citric Acid | 0.41 % | |
| | Tartaric Acid | Results are less than 0.01 % | |
| | Malic Acid | 0.20 % | |
| | Quinic Acid | 0.28 % | |
| | Succinic Acid | Results are less than 0.01 % | |
| | Lactic Acid | Results are less than 0.01 % | |
| | Glutaric Acid | 0.14 % | |
| | Acetic Acid | Results are less than 0.01 % | |

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Appendix VII. Darling 4 nutrition analyses

Final Report

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Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Email: [REDACTED] Library Number: 2016-MED-0176
Fax: PO Number: R994748

Medallion Labs Sample ID: 2016-MED-0176-02 **Customer Sample ID:** Darling 4 Minus
Assay Group **Test** **Results** **Test Date**

| | | | |
|-----------|--------------|--------------|----------|
| Calcium | Fumaric Acid | 0.01 % | |
| | Calcium | 26.3 mg/100g | 01/19/16 |
| Iron | Iron | 1.43 mg/100g | 01/19/16 |
| Sodium | Sodium | 3.44 mg/100g | 01/19/16 |
| Vitamin C | Vitamin C | 17.5 mg/100g | 01/12/16 |

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Limits of Detection and Measurement Variability are available upon request.

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Appendix VII. Darling 4 nutrition analyses

Final Report



Medallion Labs

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Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Library Number: 2016-MED-0176
PO Number: R994748

Email: [REDACTED]

Fax:

| Medallion Labs Sample ID: 2016-MED-0176-03 | | Customer Sample ID: McCabe x B3F3 | |
|--|---------------------------------------|-----------------------------------|-----------|
| Assay Group | Test | Results | Test Date |
| Sample Handling Processing Level 1 | Sample Process Fee | Sample Processed | 01/06/16 |
| ² Calories | Calories | 246 Calories/100 g | 01/25/16 |
| | Calories from Fat | 87 Calories/100 g | |
| | Calories from Saturated Fat | 12 Calories/100 g | |
| | Calories (Insoluble Fiber Subtracted) | 214 Calories/100 g | |
| ² Carbohydrates | Carbohydrates | 36.8 % | 01/25/16 |
| | Carbohydrates, Available | 28.8 % | |
| Metals Screen 1 | Metals Screen | Discount | 01/06/16 |
| Moisture by Vacuum Oven (100° C/5 hrs) | Moisture | 49.369 % | 01/12/16 |
| Ash, Overnight | Ash | 1.236 % | 01/15/16 |
| Protein, by Dumas | Protein (5.30) | 2.90 % | 01/19/16 |
| Fats, by Gas Chromatography | Total Fat | 9.71 % | 01/25/16 |
| | Saturated Fat | 1.31 % | |
| | Monounsaturated Fat | 6.42 % | |
| | cis-cis Polyunsaturated Fat | 1.55 % | |
| | trans Fat | 0.01 % | |
| Dietary Fiber | Insoluble Fiber | 8.0 % | 01/15/16 |
| | Soluble Fiber | 1.3 % | |
| | Total Fiber | 9.3 % | |
| Vitamin A Retinol | Vitamin A | Less than 50 IU/100 g | 01/17/16 |
| ² Organic Acids | Oxalic Acid | Results are less than 0.01 % | 02/01/16 |
| | Citric Acid | 0.38 % | |
| | Tartaric Acid | Results are less than 0.01 % | |
| | Malic Acid | 0.20 % | |
| | Quinic Acid | 0.31 % | |
| | Succinic Acid | Results are less than 0.01 % | |
| | Lactic Acid | Results are less than 0.01 % | |
| | Glutaric Acid | Results are less than 0.01 % | |
| | Acetic Acid | Results are less than 0.01 % | |

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Appendix VII. Darling 4 nutrition analyses

Final Report

 **Medallion Labs**

www.medallionlabs.com
1-800-245-5615 info@medlabs.com

Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Email: [REDACTED] Library Number: 2016-MED-0176
Fax: PO Number: R994748

Medallion Labs Sample ID: 2016-MED-0176-03 **Customer Sample ID:** McCabe x B3F3
Chestnuts, Peeled And Cut, Frozen

| Assay Group | Test | Results | Test Date |
|-------------|--------------|---------------|-----------|
| Calcium | Fumaric Acid | 0.01 % | |
| | Calcium | 27.8 mg/100g | 01/19/16 |
| Iron | Iron | 1.33 mg/100g | 01/19/16 |
| Sodium | Sodium | <3.00 mg/100g | 01/19/16 |
| Vitamin C | Vitamin C | 10.3 mg/100g | 01/12/16 |

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Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Library Number: 2016-MED-0176
PO Number: R994748

Email: [REDACTED]

Fax:

Medallion Labs Sample ID: 2016-MED-0176-05

Chestnuts, Peeled And Cut, Frozen

Customer Sample ID: European

| Assay Group | Test | Results | Test Date |
|--|---------------------------------------|------------------------------|-----------|
| Sample Handling Processing Level 1 | Sample Process Fee | Sample Processed | 01/06/16 |
| ² Calories | Calories | 184 Calories/100 g | 01/25/16 |
| | Calories from Fat | 12 Calories/100 g | |
| | Calories from Saturated Fat | 2 Calories/100 g | |
| | Calories (Insoluble Fiber Subtracted) | 160 Calories/100 g | |
| ² Carbohydrates | Carbohydrates | 41.4 % | 01/25/16 |
| | Carbohydrates, Available | 35.4 % | |
| Metals Screen 1 | Metals Screen | Discount | 01/06/16 |
| Moisture by Vacuum Oven (100° C/5 hrs) | Moisture | 54.609 % | 01/12/16 |
| Ash, Overnight | Ash | 1.032 % | 01/15/16 |
| Protein, by Dumas | Protein (5.30) | 1.66 % | 01/19/16 |
| Fats, by Gas Chromatography | Total Fat | 1.28 % | 01/25/16 |
| | Saturated Fat | 0.21 % | |
| | Monounsaturated Fat | 0.40 % | |
| | cis-cis Polyunsaturated Fat | 0.61 % | |
| | trans Fat | 0.00 % | |
| Dietary Fiber | Insoluble Fiber | 6.0 % | 01/15/16 |
| | Soluble Fiber | 1.2 % | |
| | Total Fiber | 7.2 % | |
| Vitamin A Retinol | Vitamin A | Less than 50 IU/100 g | 01/17/16 |
| ² Organic Acids | Oxalic Acid | Results are less than 0.01 % | 02/01/16 |
| | Citric Acid | 0.14 % | |
| | Tartaric Acid | 0.04 % | |
| | Malic Acid | 0.25 % | |
| | Quinic Acid | Results are less than 0.01 % | |
| | Succinic Acid | Results are less than 0.01 % | |
| | Lactic Acid | Results are less than 0.01 % | |
| | Glutaric Acid | Results are less than 0.01 % | |
| | Acetic Acid | Results are less than 0.01 % | |

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Final Report

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1-800-245-5615 info@medlabs.com

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Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Email: [REDACTED] Library Number: 2016-MED-0176
Fax: PO Number: R994748

Medallion Labs Sample ID: 2016-MED-0176-05 **Customer Sample ID:** European **Chestnuts, Peeled And Cut, Frozen**

| Assay Group | Test | Results | Test Date |
|-------------|--------------|--------------|-----------|
| Calcium | Fumaric Acid | 0.04 % | |
| | Calcium | 28.4 mg/100g | 01/19/16 |
| Iron | Iron | 2.21 mg/100g | 01/19/16 |
| Sodium | Sodium | 7.28 mg/100g | 01/19/16 |
| Vitamin C | Vitamin C | 32.3 mg/100g | 01/12/16 |

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Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Library Number: 2016-MED-0176
PO Number: R994748

Email: [REDACTED]

Fax:

Medallion Labs Sample ID: 2016-MED-0176-06

Chestnuts, Peeled And Cut, Frozen

Customer Sample ID: Wisconsin

| Assay Group | Test | Results | Test Date |
|--|---------------------------------------|------------------------------|-----------|
| Sample Handling Processing Level 1 | Sample Process Fee | Sample Processed | 01/06/16 |
| ² Calories | Calories | 233 Calories/100 g | 01/25/16 |
| | Calories from Fat | 64 Calories/100 g | |
| | Calories from Saturated Fat | 9 Calories/100 g | |
| | Calories (Insoluble Fiber Subtracted) | 203 Calories/100 g | |
| ² Carbohydrates | Carbohydrates | 38.0 % | 01/25/16 |
| | Carbohydrates, Available | 30.6 % | |
| Metals Screen 1 | Metals Screen | Discount | 01/06/16 |
| Moisture by Vacuum Oven (100° C/5 hrs) | Moisture | 49.424 % | 01/12/16 |
| Ash, Overnight | Ash | 1.284 % | 01/15/16 |
| Protein, by Dumas | Protein (5.30) | 4.19 % | 01/19/16 |
| Fats, by Gas Chromatography | Total Fat | 7.13 % | 01/25/16 |
| | Saturated Fat | 0.97 % | |
| | Monounsaturated Fat | 4.47 % | |
| | cis-cis Polyunsaturated Fat | 1.36 % | |
| | trans Fat | 0.01 % | |
| Dietary Fiber | Insoluble Fiber | 7.4 % | 01/15/16 |
| | Soluble Fiber | 1.7 % | |
| | Total Fiber | 9.1 % | |
| Vitamin A Retinol | Vitamin A | Less than 50 IU/100 g | 01/17/16 |
| ² Organic Acids | Oxalic Acid | Results are less than 0.01 % | 02/01/16 |
| | Citric Acid | 0.58 % | |
| | Tartaric Acid | Results are less than 0.01 % | |
| | Malic Acid | 0.16 % | |
| | Quinic Acid | 0.36 % | |
| | Succinic Acid | Results are less than 0.01 % | |
| | Lactic Acid | Results are less than 0.01 % | |
| | Glutaric Acid | Results are less than 0.01 % | |
| | Acetic Acid | Results are less than 0.01 % | |

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Final Report

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Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Email: [REDACTED] Library Number: 2016-MED-0176
Fax: PO Number: R994748

Medallion Labs Sample ID: 2016-MED-0176-06 **Customer Sample ID:** Wisconsin
Chestnuts, Peeled And Cut, Frozen

| Assay Group | Test | Results | Test Date |
|-------------|--------------|------------------------------|-----------|
| Calcium | Fumaric Acid | Results are less than 0.01 % | |
| Iron | Calcium | 63.2 mg/100g | 01/19/16 |
| Sodium | Iron | 1.34 mg/100g | 01/19/16 |
| Vitamin C | Sodium | 4.07 mg/100g | 01/19/16 |
| | Vitamin C | 13.8 mg/100g | 01/12/16 |

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Medallion Company ID: SUNY-ESF01

Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Library Number: 2016-MED-0176

PO Number: R994748

Email: [REDACTED]

Fax:

Medallion Labs Sample ID: 2016-MED-0176-07

Chestnuts, Peeled And Cut, Frozen

Customer Sample ID: Moss Lake

| Assay Group | Test | Results | Test Date |
|--|---------------------------------------|------------------------------|-----------|
| Sample Handling Processing Level 1 | Sample Process Fee | Sample Processed | 01/06/16 |
| ² Calories | Calories | 167 Calories/100 g | 01/25/16 |
| | Calories from Fat | 23 Calories/100 g | |
| | Calories from Saturated Fat | 4 Calories/100 g | |
| | Calories (Insoluble Fiber Subtracted) | 146 Calories/100 g | |
| ² Carbohydrates | Carbohydrates | 33.2 % | 01/25/16 |
| | Carbohydrates, Available | 27.9 % | |
| Metals Screen 1 | Metals Screen | Discount | 01/06/16 |
| Moisture by Vacuum Oven (100° C/5 hrs) | Moisture | 60.266 % | 01/12/16 |
| Ash, Overnight | Ash | 1.081 % | 01/15/16 |
| Protein, by Dumas | Protein (5.30) | 2.91 % | 01/19/16 |
| Fats, by Gas Chromatography | Total Fat | 2.50 % | 01/25/16 |
| | Saturated Fat | 0.40 % | |
| | Monounsaturated Fat | 1.30 % | |
| | cis-cis Polyunsaturated Fat | 0.69 % | |
| | trans Fat | 0.00 % | |
| Dietary Fiber | Insoluble Fiber | 5.3 % | 01/15/16 |
| | Soluble Fiber | 1.4 % | |
| | Total Fiber | 6.7 % | |
| Vitamin A Retinol | Vitamin A | Less than 50 IU/100 g | 01/17/16 |
| ² Organic Acids | Oxalic Acid | Results are less than 0.01 % | 02/01/16 |
| | Citric Acid | 0.34 % | |
| | Tartaric Acid | 0.09 % | |
| | Malic Acid | 0.21 % | |
| | Quinic Acid | Results are less than 0.01 % | |
| | Succinic Acid | Results are less than 0.01 % | |
| | Lactic Acid | Results are less than 0.01 % | |
| | Glutaric Acid | Results are less than 0.01 % | |
| | Acetic Acid | Results are less than 0.01 % | |

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Final Report

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Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Email: [REDACTED] Library Number: 2016-MED-0176
Fax: PO Number: R994748

Medallion Labs Sample ID: 2016-MED-0176-07 **Customer Sample ID:** Moss Lake **Chestnuts, Peeled And Cut, Frozen**

| Assay Group | Test | Results | Test Date |
|-------------|--------------|--------------|-----------|
| Calcium | Fumaric Acid | 0.02 % | |
| | Calcium | 34.6 mg/100g | 01/19/16 |
| Iron | Iron | 1.15 mg/100g | 01/19/16 |
| Sodium | Sodium | 3.08 mg/100g | 01/19/16 |
| Vitamin C | Vitamin C | 20.2 mg/100g | 01/12/16 |

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Final Report



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Medallion Company ID: SUNY-ESF01
Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Library Number: 2016-MED-0176
PO Number: R994748

Email: [REDACTED]

Fax:

| Medallion Labs Sample ID: 2016-MED-0176-08 | | Customer Sample ID: Zoar A8 | |
|--|---------------------------------------|------------------------------|-----------|
| Assay Group | Test | Results | Test Date |
| Sample Handling Processing Level 1 | Sample Process Fee | Sample Processed | 01/06/16 |
| ² Calories | Calories | 218 Calories/100 g | 01/25/16 |
| | Calories from Fat | 38 Calories/100 g | |
| | Calories from Saturated Fat | 5 Calories/100 g | |
| | Calories (Insoluble Fiber Subtracted) | 188 Calories/100 g | |
| ² Carbohydrates | Carbohydrates | 40.2 % | 01/25/16 |
| | Carbohydrates, Available | 32.6 % | |
| Metals Screen 1 | Metals Screen | Discount | 01/06/16 |
| Moisture by Vacuum Oven (100° C/5 hrs) | Moisture | 49.555 % | 01/12/16 |
| Ash, Overnight | Ash | 1.266 % | 01/15/16 |
| Protein, by Dumas | Protein (5.30) | 4.73 % | 01/19/16 |
| Fats, by Gas Chromatography | Total Fat | 4.25 % | 01/25/16 |
| | Saturated Fat | 0.56 % | |
| | Monounsaturated Fat | 2.68 % | |
| | cis-cis Polyunsaturated Fat | 0.83 % | |
| | trans Fat | 0.00 % | |
| Dietary Fiber | Insoluble Fiber | 7.6 % | 01/15/16 |
| | Soluble Fiber | 1.5 % | |
| | Total Fiber | 9.1 % | |
| Vitamin A Retinol | Vitamin A | Less than 50 IU/100 g | 01/17/16 |
| ² Organic Acids | Oxalic Acid | Results are less than 0.01 % | 02/01/16 |
| | Citric Acid | 0.68 % | |
| | Tartaric Acid | Results are less than 0.01 % | |
| | Malic Acid | 0.16 % | |
| | Quinic Acid | 0.38 % | |
| | Succinic Acid | Results are less than 0.01 % | |
| | Lactic Acid | Results are less than 0.01 % | |
| | Glutaric Acid | Results are less than 0.01 % | |
| | Acetic Acid | Results are less than 0.01 % | |

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Company Code: 16311

Andrew Newhouse
SUNF-ESF
1 Forestry Drive Bray Hall
Syracuse, NY 13210

Library Number: 2016-MED-0176

PO Number: R994748

Email: [REDACTED]

Fax:

Medallion Labs Sample ID: 2016-MED-0176-08

Chestnuts, Peeled And Cut, Frozen

Customer Sample ID: Zoar A8

| Assay Group | Test | Results | Test Date |
|-------------|--------------|------------------------------|-----------|
| Calcium | Fumaric Acid | Results are less than 0.01 % | |
| Iron | Calcium | 63.4 mg/100g | 01/19/16 |
| Sodium | Iron | 1.37 mg/100g | 01/19/16 |
| Vitamin C | Sodium | 3.63 mg/100g | 01/19/16 |
| | Vitamin C | 22.8 mg/100g | 01/12/16 |

Results Approved By: Leena Babrekar

(Authorized Reviewer)

Method References:

| Assay Group | Method Reference |
|--|---------------------------------------|
| Ash, Overnight | AOAC: 923.03 |
| Calcium | AOAC: 975.03, 985.01, 984.27, 2011.14 |
| Calories | Determined through calculation |
| Carbohydrates | Determined through calculation |
| Dietary Fiber | AOAC: 991.43* |
| Fats, by Gas Chromatography | AOAC: 996.06* |
| Iron | AOAC: 975.03, 985.01, 984.27, 2011.14 |
| Moisture by Vacuum Oven (100° C/5 hrs) | AOAC: 926.08, 927.05 |
| Organic Acids | AOAC: 986.13 |
| Protein, by Dumas | AOAC: 992.15; AACC: 46-30 |
| Sodium | AOAC: 975.03, 985.01, 984.27, 2011.14 |
| Vitamin A Retinol | AOAC: 2001.13; AACC: 86-06 |
| Vitamin C | AOAC: 967.22, 984.26* |

* This method has been modified.

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Appendix VII. Darling 4 nutrition analyses

Fatty Acid Profile



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www.medallionlabs.com
1-800-245-5615 info@medlabs.com
Andrew Newhouse
SUNF-ESF
Email: [REDACTED]

Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311
Library: 2016-MED-0176
PO Number: R994748
Fax:

| | | | | | |
|--------------|-----------------------------------|-------------|------------|----------------|------------|
| Sample ID: | 2016-MED-0176-01 | Identifier: | Darling 4+ | Date Reviewed: | 02/02/2016 |
| Description: | Chestnuts, Peeled And Cut, Frozen | | | | |
| Analysis: | Triglycerides | | Date Run: | 01/25/2016 | |

| Component Name | Normalized by Weight | % (w/w) as Triglyceride in Product | % (w/w) Fatty Acids in Product | | | |
|--|----------------------|------------------------------------|--------------------------------|-----------------------------|-------------------------------------|-------------------------------|
| | | | Saturated Fatty Acids | Monounsaturated Fatty Acids | cis-cis Polyunsaturated Fatty Acids | trans Unsaturated Fatty Acids |
| 4:0 Butyric | | | | | | |
| 6:0 Caproic | | | | | | |
| 8:0 Caprylic | | | | | | |
| 10:0 Capric | | | | | | |
| 12:0 Lauric | 0.032% | 0.003 | 0.003 | | | |
| 13:0 Tridecanoic | | | | | | |
| 14:0 Myristic | 0.085% | 0.008 | 0.008 | | | |
| 14:1 t-Tetradecanoic | | | | | | |
| 14:1 Myristoleic | | | | | | |
| 15:0 Pentadecanoic | 0.021% | 0.002 | 0.002 | | | |
| 15:1 Pentadecenoic | | | | | | |
| 16:0 Palmitic | 11.442% | 1.081 | 1.030 | | | |
| 16:1 t-Hexadecenoic | 0.074% | 0.007 | | | | 0.007 |
| 16:1 Palmitoleic | 0.042% | 0.004 | | 0.004 | | |
| 17:0 Margaric | 0.106% | 0.010 | 0.010 | | | |
| 17:1 Margaroleic | 0.042% | 0.004 | | 0.004 | | |
| 18:0 Stearic | 1.831% | 0.173 | 0.166 | | | |
| 18:1 trans-Elaidic | | | | | | |
| 18:1 Oleic | 65.686% | 6.206 | | 5.939 | | |
| 18:2 t-Octadecadienoic | 0.074% | 0.007 | | | | 0.007 |
| 18:2 Linoleic | 16.850% | 1.592 | | | 1.523 | |
| 20:0 Arachidic | 0.603% | 0.057 | 0.055 | | | |
| 18:3 g-Linolenic | | | | | | |
| 20:1 Gadoleic | 0.900% | 0.085 | | 0.082 | | |
| 18:3 Linolenic | 1.810% | 0.171 | | | 0.164 | |
| 21:0 Heneicosanoic | | | | | | |
| 18:2 conjugated-Linoleic | | | | | | |
| 18:4 Octadecatetraenoic | | | | | | |
| 20:2 Eicosadienoic | 0.053% | 0.005 | | | 0.005 | |
| 22:0 Behenic | 0.233% | 0.022 | 0.021 | | | |
| 20:3 g-Eicosatrienoic | | | | | | |
| 22:1 Erucic | | | | | | |
| 20:3 Eicosatrienoic | | | | | | |
| 20:4 Arachidonic | | | | | | |
| 23:0 Ticosanoic | | | | | | |
| 22:2 Docosadienoic | | | | | | |
| 24:0 Lignoceric | 0.116% | 0.011 | 0.011 | | | |
| 20:5 Eicosapentaenoic | | | | | | |
| 24:1 Nervonic | | | | | | |
| 22:3 Docosatrienoic | | | | | | |
| 22:4 Docosatetraenoic | | | | | | |
| 22:5 Docosapentaenoic | | | | | | |
| 22:6 Docosahexaenoic | | | | | | |
| Totals: | 100.00% | 9.45 | 1.30 | 6.03 | 1.69 | 0.01 |
| Percent of Fatty Acid Components based on Total Fat: | | | 14.47% | 66.67% | 18.71% | 0.15% |
| Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA): | | | | | 0.00% | |

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Appendix VII. Darling 4 nutrition analyses

Fatty Acid Profile



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www.medallionlabs.com
1-800-245-5615 info@medlabs.com
Andrew Newhouse
SUNF-ESF
Email: [REDACTED]

Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311
Library: 2016-MED-0176
PO Number: R994748
Fax:

| | | | | | |
|--------------|-----------------------------------|-------------|-----------------|----------------|------------|
| Sample ID: | 2016-MED-0176-02 | Identifier: | Darling 4 Minus | Date Reviewed: | 02/02/2016 |
| Description: | Chestnuts, Peeled And Cut, Frozen | | | | |
| Analysis: | Triglycerides | | Date Run: | 01/25/2016 | |

| Component Name | Normalized by Weight | % (w/w) as Triglyceride in Product | % (w/w) Fatty Acids in Product | | | |
|--|----------------------|------------------------------------|--------------------------------|-----------------------------|-------------------------------------|-------------------------------|
| | | | Saturated Fatty Acids | Monounsaturated Fatty Acids | cis-cis Polyunsaturated Fatty Acids | trans Unsaturated Fatty Acids |
| 4:0 Butyric | | | | | | |
| 6:0 Caproic | | | | | | |
| 8:0 Caprylic | | | | | | |
| 10:0 Capric | 0.011% | 0.001 | 0.001 | | | |
| 12:0 Lauric | 0.034% | 0.003 | 0.003 | | | |
| 13:0 Tridecanoic | | | | | | |
| 14:0 Myristic | 0.092% | 0.008 | 0.008 | | | |
| 14:1 t-Tetradecanoic | | | | | | |
| 14:1 Myristoleic | | | | | | |
| 15:0 Pentadecanoic | 0.023% | 0.002 | 0.002 | | | |
| 15:1 Pentadenoic | | | | | | |
| 16:0 Palmitic | 11.352% | 0.990 | 0.943 | | | |
| 16:1 t-Hexadecenoic | 0.069% | 0.006 | | | | 0.006 |
| 16:1 Palmitoleic | 0.046% | 0.004 | | 0.004 | | |
| 17:0 Margaric | 0.103% | 0.009 | 0.009 | | | |
| 17:1 Margaroleic | 0.034% | 0.003 | | 0.003 | | |
| 18:0 Stearic | 1.766% | 0.154 | 0.147 | | | |
| 18:1 trans-Elaidic | 0.034% | 0.003 | | | | 0.003 |
| 18:1 Oleic | 66.093% | 5.764 | | 5.516 | | |
| 18:2 t-Octadecadienoic | 0.057% | 0.005 | | | | 0.005 |
| 18:2 Linoleic | 16.489% | 1.438 | | | 1.376 | |
| 20:0 Arachidic | 0.596% | 0.052 | 0.050 | | | |
| 18:3 g-Linolenic | | | | | | |
| 20:1 Gadoleic | 0.906% | 0.079 | | 0.076 | | |
| 18:3 Linolenic | 1.823% | 0.159 | | | 0.152 | |
| 21:0 Heneicosanoic | | | | | | |
| 18:2 conjugated-Linoleic | | | | | | |
| 18:4 Octadecatetraenoic | | | | | | |
| 20:2 Eicosadienoic | 0.080% | 0.007 | | | 0.007 | |
| 22:0 Behenic | 0.252% | 0.022 | 0.021 | | | |
| 20:3 g-Eicosatrienoic | | | | | | |
| 22:1 Erucic | | | | | | |
| 20:3 Eicosatrienoic | | | | | | |
| 20:4 Arachidonic | | | | | | |
| 23:0 Ticosanoic | | | | | | |
| 22:2 Docosadienoic | | | | | | |
| 24:0 Lignoceric | 0.138% | 0.012 | 0.012 | | | |
| 20:5 Eicosapentaenoic | | | | | | |
| 24:1 Nervonic | | | | | | |
| 22:3 Docosatrienoic | | | | | | |
| 22:4 Docosatetraenoic | | | | | | |
| 22:5 Docosapentaenoic | | | | | | |
| 22:6 Docosahexaenoic | | | | | | |
| Totals: | 100.00% | 8.72 | 1.20 | 5.60 | 1.53 | 0.01 |
| Percent of Fatty Acid Components based on Total Fat: | | | 14.37% | 67.08% | 18.39% | 0.16% |
| Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA): | | | | | 0.00% | |

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Appendix VII. Darling 4 nutrition analyses

Fatty Acid Profile



Medallion Labs

www.medallionlabs.com
1-800-245-5615 info@medlabs.com
Andrew Newhouse
SUNY-ESF
Email: [REDACTED]

Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311
Library: 2016-MED-0176
PO Number: R994748
Fax:

| | | | | | |
|------------|------------------|-------------|---------------|----------------|------------|
| Sample ID: | 2016-MED-0176-03 | Identifier: | McCabe x B3F3 | Date Reviewed: | 02/02/2016 |
|------------|------------------|-------------|---------------|----------------|------------|

Description: Chestnuts, Peeled And Cut, Frozen

Analysis: Triglycerides

Date Run: 01/25/2016

| Component Name | Normalized by Weight | % (w/w) as Triglyceride in Product | % (w/w) Fatty Acids in Product | | | |
|--|----------------------|------------------------------------|--------------------------------|-----------------------------|-------------------------------------|-------------------------------|
| | | | Saturated Fatty Acids | Monounsaturated Fatty Acids | cis-cis Polyunsaturated Fatty Acids | trans Unsaturated Fatty Acids |
| 4:0 Butyric | | | | | | |
| 6:0 Caproic | | | | | | |
| 8:0 Caprylic | | | | | | |
| 10:0 Capric | | | | | | |
| 12:0 Lauric | | | | | | |
| 13:0 Tridecanoic | | | | | | |
| 14:0 Myristic | | | | | | |
| 14:1 t-Tetradecanoic | | | | | | |
| 14:1 Myristoleic | | | | | | |
| 15:0 Pentadecanoic | | | | | | |
| 15:1 Pentadenoic | | | | | | |
| 16:0 Palmitic | 10.941% | 1.062 | 1.012 | | | |
| 16:1 t-Hexadecenoic | 0.062% | 0.006 | | | | 0.006 |
| 16:1 Palmitoleic | 0.041% | 0.004 | | 0.004 | | |
| 17:0 Margaric | 0.113% | 0.011 | 0.011 | | | |
| 17:1 Margaroleic | 0.062% | 0.006 | | 0.006 | | |
| 18:0 Stearic | 1.906% | 0.185 | 0.177 | | | |
| 18:1 trans-Elaidic | | | | | | |
| 18:1 Oleic | 68.054% | 6.606 | | 6.322 | | |
| 18:2 t-Octadecadienoic | 0.031% | 0.003 | | | | 0.003 |
| 18:2 Linoleic | 14.948% | 1.451 | | | | 1.388 |
| 20:0 Arachidic | 0.659% | 0.064 | 0.062 | | | |
| 18:3 g-Linolenic | | | | | | |
| 20:1 Gadoleic | 0.927% | 0.090 | | 0.086 | | |
| 18:3 Linolenic | 1.700% | 0.165 | | | | 0.158 |
| 21:0 Heneicosanoic | 0.031% | 0.003 | 0.003 | | | |
| 18:2 conjugated-Linoleic | | | | | | |
| 18:4 Octadecatetraenoic | | | | | | |
| 20:2 Eicosadienoic | 0.072% | 0.007 | | | | 0.007 |
| 22:0 Behenic | 0.268% | 0.026 | 0.025 | | | |
| 20:3 g-Eicosatrienoic | | | | | | |
| 22:1 Erucic | 0.010% | 0.001 | | 0.001 | | |
| 20:3 Eicosatrienoic | | | | | | |
| 20:4 Arachidonic | | | | | | |
| 23:0 Tricosanoic | 0.041% | 0.004 | 0.004 | | | |
| 22:2 Docosadienoic | | | | | | |
| 24:0 Lignoceric | 0.134% | 0.013 | 0.013 | | | |
| 20:5 Eicosapentaenoic | | | | | | |
| 24:1 Nervonic | | | | | | |
| 22:3 Docosatrienoic | | | | | | |
| 22:4 Docosatetraenoic | | | | | | |
| 22:5 Docosapentaenoic | | | | | | |
| 22:6 Docosahexaenoic | | | | | | |
| Totals: | 100.00% | 9.71 | 1.31 | 6.42 | 1.55 | 0.01 |
| Percent of Fatty Acid Components based on Total Fat: | | | 14.09% | 69.09% | 16.72% | 0.09% |
| Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA): | | | | | 0.00% | |

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Medallion Labs 9000 Plymouth Ave. N., Minneapolis, MN 55427

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Appendix VII. Darling 4 nutrition analyses

Fatty Acid Profile



Medallion Labs

www.medallionlabs.com
1-800-245-5615 info@medlabs.com
Andrew Newhouse
SUNF-ESF
Email: [REDACTED]

Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311
Library: 2016-MED-0176
PO Number: R994748
Fax:

| | | | | | |
|------------|------------------|-------------|----------|----------------|------------|
| Sample ID: | 2016-MED-0176-05 | Identifier: | European | Date Reviewed: | 02/02/2016 |
|------------|------------------|-------------|----------|----------------|------------|

Description: Chestnuts, Peeled And Cut, Frozen

Analysis: Triglycerides

Date Run: 01/25/2016

| Component Name | Normalized by Weight | % (w/w) as Triglyceride in Product | % (w/w) Fatty Acids in Product | | | |
|--|----------------------|------------------------------------|--------------------------------|-----------------------------|-------------------------------------|-------------------------------|
| | | | Saturated Fatty Acids | Monounsaturated Fatty Acids | cis-cis Polyunsaturated Fatty Acids | trans Unsaturated Fatty Acids |
| 4:0 Butyric | | | | | | |
| 6:0 Caproic | | | | | | |
| 8:0 Caprylic | | | | | | |
| 10:0 Capric | | | | | | |
| 12:0 Lauric | 0.157% | 0.002 | 0.002 | | | |
| 13:0 Tridecanoic | | | | | | |
| 14:0 Myristic | | | | | | |
| 14:1 t-Tetradecanoic | | | | | | |
| 14:1 Myristoleic | | | | | | |
| 15:0 Pentadecanoic | | | | | | |
| 15:1 Pentadenoic | | | | | | |
| 16:0 Palmitic | 15.047% | 0.192 | 0.183 | | | |
| 16:1 t-Hexadecenoic | 0.157% | 0.002 | | | | 0.002 |
| 16:1 Palmitoleic | 0.470% | 0.006 | | 0.006 | | |
| 17:0 Margaric | 0.078% | 0.001 | 0.001 | | | |
| 17:1 Margaroleic | | | | | | |
| 18:0 Stearic | 1.176% | 0.015 | 0.014 | | | |
| 18:1 trans-Elaidic | | | | | | |
| 18:1 Oleic | 31.818% | 0.406 | | 0.389 | | |
| 18:2 t-Octadecadienoic | 0.078% | 0.001 | | | | 0.001 |
| 18:2 Linoleic | 43.417% | 0.554 | | | 0.530 | |
| 20:0 Arachidic | 0.313% | 0.004 | 0.004 | | | |
| 18:3 g-Linolenic | | | | | | |
| 20:1 Gadoleic | 0.470% | 0.006 | | 0.006 | | |
| 18:3 Linolenic | 6.191% | 0.079 | | | 0.076 | |
| 21:0 Heneicosanoic | | | | | | |
| 18:2 conjugated-Linoleic | | | | | | |
| 18:4 Octadecatetraenoic | | | | | | |
| 20:2 Eicosadienoic | | | | | | |
| 22:0 Behenic | 0.313% | 0.004 | 0.004 | | | |
| 20:3 g-Eicosatrienoic | | | | | | |
| 22:1 Erucic | | | | | | |
| 20:3 Eicosatrienoic | | | | | | |
| 20:4 Arachidonic | | | | | | |
| 23:0 Tricosanoic | 0.078% | 0.001 | 0.001 | | | |
| 22:2 Docosadienoic | | | | | | |
| 24:0 Lignoceric | 0.235% | 0.003 | 0.003 | | | |
| 20:5 Eicosapentaenoic | | | | | | |
| 24:1 Nervonic | | | | | | |
| 22:3 Docosatrienoic | | | | | | |
| 22:4 Docosatetraenoic | | | | | | |
| 22:5 Docosapentaenoic | | | | | | |
| 22:6 Docosahexaenoic | | | | | | |
| Totals: | 100.00% | 1.28 | 0.21 | 0.40 | 0.61 | 0.00 |
| Percent of Fatty Acid Components based on Total Fat: | | | 17.40% | 32.76% | 49.61% | 0.24% |
| Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA): | | | | | 0.00% | |

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Medallion Labs 9000 Plymouth Ave. N., Minneapolis, MN 55427

Page 4 of 7

Appendix VII. Darling 4 nutrition analyses

Fatty Acid Profile



Medallion Labs

www.medallionlabs.com
1-800-245-5615 info@medlabs.com
Andrew Newhouse
SUNF-ESF

Email: [REDACTED]

Completion Date: February 02, 2016

Date Submitted: January 06, 2016

Medallion Company ID: SUNY-ESF01

Company Code: 16311

Library: 2016-MED-0176

PO Number: R994748

Fax:

Sample ID: 2016-MED-0176-06 Identifier: Wisconsin Date Reviewed: 02/02/2016

Description: Chestnuts, Peeled And Cut, Frozen Analysis: Triglycerides Date Run: 01/25/2016

| Component Name | Normalized by Weight | % (w/w) as Triglyceride in Product | % (w/w) Fatty Acids in Product | | | |
|--|----------------------|------------------------------------|--------------------------------|-----------------------------|-------------------------------------|-------------------------------|
| | | | Saturated Fatty Acids | Monounsaturated Fatty Acids | cis-cis Polyunsaturated Fatty Acids | trans Unsaturated Fatty Acids |
| 4:0 Butyric | | | | | | |
| 6:0 Caproic | | | | | | |
| 8:0 Caprylic | | | | | | |
| 10:0 Capric | | | | | | |
| 12:0 Lauric | 0.014% | 0.001 | 0.001 | | | |
| 13:0 Tridecanoic | | | | | | |
| 14:0 Myristic | 0.084% | 0.006 | 0.006 | | | |
| 14:1 t-Tetradecanoic | | | | | | |
| 14:1 Myristoleic | | | | | | |
| 15:0 Pentadecanoic | 0.028% | 0.002 | 0.002 | | | |
| 15:1 Pentadecenoic | | | | | | |
| 16:0 Palmitic | 11.699% | 0.834 | 0.795 | | | |
| 16:1 t-Hexadecenoic | 0.070% | 0.005 | | | | 0.005 |
| 16:1 Palmitoleic | 0.056% | 0.004 | | 0.004 | | |
| 17:0 Margaric | 0.084% | 0.006 | 0.006 | | | |
| 17:1 Margaroleic | 0.028% | 0.002 | | 0.002 | | |
| 18:0 Stearic | 1.417% | 0.101 | 0.097 | | | |
| 18:1 trans-Elaidic | | | | | | |
| 18:1 Oleic | 64.539% | 4.601 | | 4.403 | | |
| 18:2 t-Octadecadienoic | 0.042% | 0.003 | | | | 0.003 |
| 18:2 Linoleic | 18.320% | 1.306 | | | | 1.249 |
| 20:0 Arachidic | 0.505% | 0.036 | 0.035 | | | |
| 18:3 g-Linolenic | | | | | | |
| 20:1 Gadoleic | 0.926% | 0.066 | | 0.063 | | |
| 18:3 Linolenic | 1.613% | 0.115 | | | | 0.110 |
| 21:0 Heneicosanoic | 0.028% | 0.002 | 0.002 | | | |
| 18:2 conjugated-Linoleic | 0.014% | 0.001 | | | | |
| 18:4 Octadecatetraenoic | | | | | | |
| 20:2 Eicosadienoic | 0.056% | 0.004 | | | | 0.004 |
| 22:0 Behenic | 0.267% | 0.019 | 0.018 | | | |
| 20:3 g-Eicosatrienoic | | | | | | |
| 22:1 Erucic | 0.014% | 0.001 | | 0.001 | | |
| 20:3 Eicosatrienoic | | | | | | |
| 20:4 Arachidonic | | | | | | |
| 23:0 Tricosanoic | 0.042% | 0.003 | 0.003 | | | |
| 22:2 Docosadienoic | | | | | | |
| 24:0 Lignoceric | 0.154% | 0.011 | 0.011 | | | |
| 20:5 Eicosapentaenoic | | | | | | |
| 24:1 Nervonic | | | | | | |
| 22:3 Docosatrienoic | | | | | | |
| 22:4 Docosatetraenoic | | | | | | |
| 22:5 Docosapentaenoic | | | | | | |
| 22:6 Docosahexaenoic | | | | | | |
| Totals: | 100.00% | 7.13 | 0.97 | 4.47 | 1.36 | 0.01 |
| Percent of Fatty Acid Components based on Total Fat: | | | 14.32% | 65.56% | 19.99% | 0.11% |
| Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA): | | | | | 0.00% | |

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Appendix VII. Darling 4 nutrition analyses

Fatty Acid Profile



Medallion Labs

www.medallionlabs.com
1-800-245-5615 info@medlabs.com
Andrew Newhouse
SUNF-ESF
Email: [REDACTED]

Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311
Library: 2016-MED-0176
PO Number: R994748
Fax:

| | | | | | |
|--------------|-----------------------------------|-------------|-----------|----------------|------------|
| Sample ID: | 2016-MED-0176-07 | Identifier: | Moss Lake | Date Reviewed: | 02/02/2016 |
| Description: | Chestnuts, Peeled And Cut, Frozen | | | | |
| Analysis: | Triglycerides | | Date Run: | 01/25/2016 | |

| Component Name | Normalized by Weight | % (w/w) as Triglyceride in Product | % (w/w) Fatty Acids in Product | | | |
|--|----------------------|------------------------------------|--------------------------------|-----------------------------|-------------------------------------|-------------------------------|
| | | | Saturated Fatty Acids | Monounsaturated Fatty Acids | cis-cis Polyunsaturated Fatty Acids | trans Unsaturated Fatty Acids |
| 4:0 Butyric | | | | | | |
| 6:0 Caproic | | | | | | |
| 8:0 Caprylic | | | | | | |
| 10:0 Capric | | | | | | |
| 12:0 Lauric | | | | | | |
| 13:0 Tridecanoic | | | | | | |
| 14:0 Myristic | | | | | | |
| 14:1 t-Tetradecanoic | | | | | | |
| 14:1 Myristoleic | | | | | | |
| 15:0 Pentadecanoic | | | | | | |
| 15:1 Pentadenoic | | | | | | |
| 16:0 Palmitic | | | | | | |
| 16:1 t-Hexadecenoic | | | | | | |
| 16:1 Palmitoleic | | | | | | |
| 17:0 Margaric | | | | | | |
| 17:1 Margaroleic | | | | | | |
| 18:0 Stearic | 1.838% | 0.046 | 0.044 | | | |
| 18:1 trans-Elaidic | | | | | | |
| 18:1 Oleic | 53.895% | 1.349 | | 1.291 | | |
| 18:2 t-Octadecadienoic | | | | | | |
| 18:2 Linoleic | 25.410% | 0.636 | | | 0.608 | |
| 20:0 Arachidic | 0.439% | 0.011 | 0.011 | | | |
| 18:3 g-Linolenic | | | | | | |
| 20:1 Gadoleic | 0.519% | 0.013 | | 0.012 | | |
| 18:3 Linolenic | 3.596% | 0.090 | | | 0.086 | |
| 21:0 Heneicosanoic | | | | | | |
| 18:2 conjugated-Linoleic | | | | | | |
| 18:4 Octadecatetraenoic | | | | | | |
| 20:2 Eicosadienoic | | | | | | |
| 22:0 Behenic | 0.280% | 0.007 | 0.007 | | | |
| 20:3 g-Eicosatrienoic | | | | | | |
| 22:1 Erucic | | | | | | |
| 20:3 Eicosatrienoic | | | | | | |
| 20:4 Arachidonic | | | | | | |
| 23:0 Ticosanoic | | | | | | |
| 22:2 Docosadienoic | | | | | | |
| 24:0 Lignoceric | 0.160% | 0.004 | 0.004 | | | |
| 20:5 Eicosapentaenoic | | | | | | |
| 24:1 Nervonic | | | | | | |
| 22:3 Docosatrienoic | | | | | | |
| 22:4 Docosatetraenoic | | | | | | |
| 22:5 Docosapentaenoic | | | | | | |
| 22:6 Docosahexaenoic | | | | | | |
| Totals: | 100.00% | 2.50 | 0.40 | 1.30 | 0.69 | 0.00 |
| Percent of Fatty Acid Components based on Total Fat: | | | 16.58% | 54.41% | 29.01% | 0.00% |
| Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA): | | | | | 0.00% | |

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Fatty Acid Profile



Medallion Labs

www.medallionlabs.com
1-800-245-5615 info@medlabs.com
Andrew Newhouse
SUNF-ESF
Email: [REDACTED]

Completion Date: February 02, 2016
Date Submitted: January 06, 2016
Medallion Company ID: SUNY-ESF01
Company Code: 16311
Library: 2016-MED-0176
PO Number: R994748
Fax:

| Sample ID: | 2016-MED-0176-01 | Identifier: | Darling 4+ | Date Reviewed: | 02/02/2016 | |
|--|-----------------------------------|------------------------------------|-----------------------|-----------------------------|-------------------------------------|-------------|
| Description: | Chestnuts, Peeled And Cut, Frozen | Date Run: | 01/25/2016 | | | |
| Analysis: | Triglycerides | % (w/w) Fatty Acids in Product | | | | |
| Component Name | Normalized by Weight | % (w/w) as Triglyceride in Product | Saturated Fatty Acids | Monounsaturated Fatty Acids | cis-cis Polyunsaturated Fatty Acids | |
| | | | | | trans Unsaturated Fatty Acids | |
| 4:0 Butyric | | | | | | |
| 6:0 Caproic | | | | | | |
| 8:0 Caprylic | | | | | | |
| 10:0 Capric | | | | | | |
| 12:0 Lauric | 0.032% | 0.003 | 0.003 | | | |
| 13:0 Tridecanoic | | | | | | |
| 14:0 Myristic | 0.085% | 0.008 | 0.008 | | | |
| 14:1 t-Tetradecanoic | | | | | | |
| 14:1 Myristoleic | | | | | | |
| 15:0 Pentadecanoic | 0.021% | 0.002 | 0.002 | | | |
| 15:1 Pentadecenoic | | | | | | |
| 16:0 Palmitic | 11.442% | 1.081 | 1.030 | | | |
| 16:1 t-Hexadecenoic | 0.074% | 0.007 | | | 0.007 | |
| 16:1 Palmitoleic | 0.042% | 0.004 | | 0.004 | | |
| 17:0 Margaric | 0.106% | 0.010 | 0.010 | | | |
| 17:1 Margaroleic | 0.042% | 0.004 | | 0.004 | | |
| 18:0 Stearic | 1.831% | 0.173 | 0.166 | | | |
| 18:1 trans-Elaidic | | | | | | |
| 18:1 Oleic | 65.686% | 6.206 | | 5.939 | | |
| 18:2 t-Octadecadienoic | 0.074% | 0.007 | | | 0.007 | |
| 18:2 Linoleic | 16.850% | 1.592 | | | 1.523 | |
| 20:0 Arachidic | 0.603% | 0.057 | 0.055 | | | |
| 18:3 g-Linolenic | | | | | | |
| 20:1 Gadoleic | 0.900% | 0.085 | | 0.082 | | |
| 18:3 Linolenic | 1.810% | 0.171 | | | 0.164 | |
| 21:0 Heneicosanoic | | | | | | |
| 18:2 conjugated-Linoleic | | | | | | |
| 18:4 Octadecatetraenoic | | | | | | |
| 20:2 Eicosadienoic | 0.053% | 0.005 | | | 0.005 | |
| 22:0 Behenic | 0.233% | 0.022 | 0.021 | | | |
| 20:3 g-Eicosatrienoic | | | | | | |
| 22:1 Erucic | | | | | | |
| 20:3 Eicosatrienoic | | | | | | |
| 20:4 Arachidononic | | | | | | |
| 23:0 Tricosanoic | | | | | | |
| 22:2 Docosadienoic | | | | | | |
| 24:0 Lignoceric | 0.116% | 0.011 | 0.011 | | | |
| 20:5 Eicosapentaenoic | | | | | | |
| 24:1 Nervonic | | | | | | |
| 22:3 Docosatrienoic | | | | | | |
| 22:4 Docosatetraenoic | | | | | | |
| 22:5 Docosapentaenoic | | | | | | |
| 22:6 Docosahexaenoic | | | | | | |
| Totals: | 100.00% | 9.45 | 1.30 | 6.03 | 1.69 | 0.01 |
| Percent of Fatty Acid Components based on Total Fat: | | | 14.47% | 66.67% | 18.71% | 0.15% |
| Omega-3 Fatty Acids, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA): | | | | | | 0.00% |

Medallion's services, including this report, are provided subject to all provisions of Medallion's Standard Terms and Conditions, a copy of which appears at www.medlabs.com.
 Limits of Detection, Method References and Measurement Variability are available upon request.

Date Issued: 2/2/2016 Medallion Labs 9000 Plymouth Ave. N., Minneapolis, MN 55427 Page 1 of 7

Appendix VIII. *Tannin analysis – peeled chestnuts*

Natural Product Analysis Lab-FST

1901 N 21st Street, Lincoln, NE 68588-6205

University of Nebraska-Lincoln
Department of Food Science and Technology

Certificate of Analysis

| | |
|-------------------------------|----------------------------------|
| Product: Chestnuts | No. of Samples: 6 samples |
| Analysis: Tannins | Job No.: AB171 |
| Company: Andy Newhouse | |

| Sample | Tannins (mg per g) |
|----------|-----------------------|
| ACS - NT | 0.111 +/- 0.012 |
| ACS + T | 0.133 +/- 0.014 |
| CHN | 0.169 +/- 0.030 |
| F9 - NT | 0.199 +/- 0.032 |
| F9 + T | 0.199 +/- 0.025 |
| MLAC | 0.112 +/- 0.009 |

Raw data from above

| Sample | Tannins (mg per g) |
|------------|-----------------------|
| ACS - NT 1 | 0.097 |
| ACS - NT 2 | 0.120 |
| ACS - NT 3 | 0.116 |
| ACS + T 1 | 0.131 |
| ACS + T 2 | 0.148 |
| ACS + T 3 | 0.120 |
| CHN 1 | 0.171 |
| CHN 2 | 0.198 |
| CHN 3 | 0.137 |
| F9 - NT 1 | 0.174 |
| F9 - NT 2 | 0.235 |
| F9 - NT 3 | 0.187 |
| F9 + T 1 | 0.170 |
| F9 + T 2 | 0.217 |
| F9 + T 3 | 0.209 |
| MLAC 1 | 0.103 |
| MLAC 2 | 0.120 |
| MLAC 3 | 0.113 |

Appendix VIII. *Tannin analysis – peeled chestnuts*

Natural Product Analysis Lab-FST

1901 N 21st Street, Lincoln, NE 68588-6205

University of Nebraska-Lincoln

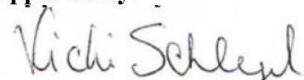
Department of Food Science and Technology

Comments: Results expressed as the mean +/- standard deviation triplicate analyses.

Tannins: Acidified vanillin method with vertical extraction using an external standard.

Vannilin assay after samples were vertically extracted.

Approved by:



Date: 2-17-2019

Vicki Schlegel, Ph.D, Analytical Laboratory Supervisor, Associate Professor
Department of Food Science and Technology University of Nebraska – Lincoln
Lincoln NE 68588-6205

Natural Product Analysis Lab-FST

1901 N 21st Street, Lincoln, NE 68588-6205

University of Nebraska-Lincoln

Department of Food Science and Technology

Certificate of Analysis

Product: Chestnuts

No. of Samples: 6 samples

Analysis: Tannins

Job No.: AB177

Company: Andy Newhouse

| Sample | Tannins (mg per g) |
|---------|-----------------------|
| F9 + T | 1.70 +/- 0.31 |
| F9 - NT | 1.70 +/- 0.42 |
| SHERB | 1.65 +/- 0.47 |
| MLAC | 0.15 +/- 0.03 |
| CHN | 0.45 +/- 0.06 |
| MASS | 2.59 +/- 0.34 |

Raw data from above results

| Sample | Tannins (mg per g) |
|-----------|-----------------------|
| F9 + T 1 | 1.34 |
| F9 + T 2 | 1.93 |
| F9 + T 3 | 1.82 |
| F9 - NT 1 | 1.36 |
| F9 - NT 2 | 1.56 |
| F9 - NT 3 | 2.17 |
| SHERB 1 | 1.82 |
| SHERB 2 | 1.12 |
| SHERB 3 | 2.01 |
| MLAC 1 | 0.18 |
| MLAC 2 | 0.14 |
| MLAC 3 | 0.12 |
| CHN 1 | 0.52 |
| CHN 2 | 0.40 |
| CHN 3 | 0.42 |
| MASS 1 | 2.93 |
| MASS 2 | 2.58 |
| MASS 3 | 2.25 |

Natural Product Analysis Lab-FST

1901 N 21st Street, Lincoln, NE 68588-6205

University of Nebraska-Lincoln

Department of Food Science and Technology

Comments: Results expressed as the mean +/- standard deviation triplicate analyses.

Tannins: Acidified vanillin method with vertical extraction using an external standard.

Vannilin assay after samples were vertically extracted.

Approved by:



Date: 2-17-2019

Vicki Schlegel, Ph.D, Analytical Laboratory Supervisor, Associate Professor
Department of Food Science and Technology University of Nebraska – Lincoln
Lincoln NE 68588-6205

Appendix IX. Sequence of pTACF3 T-DNA in Darling 4

T-DNA sequence of pTACF3 vector; i.e. new DNA used to transform Darling 4. Table shows locations of relevant genetic elements, PCR primer binding sites, and restriction sites used in Southern hybridization. (See Figures 7.1a and 10.4.1a for schematic representation and orientation of genetic elements.) PCR primer binding sites are shown in Appendix III (Darling 58); not repeated here. Relevance of Darling 4 for copy number analysis and other bridging data to Darling 58 is described in Section 10. Darling 4 also includes the pGFP vector; not shown here.

| Base Pair Location | pTACF3 Genetic Element (see Section 10.4.1) |
|--------------------|---|
| 1 – 22 | Right Border (<i>italic</i>) |
| 235 – 1202 | <u>VspB Promoter</u> (underlined) |
| 1203 – 1874 | Oxalate Oxidase (highlighted green) |
| 1875 – 2644 | ACTII Terminator (bold) |
| 2645 – 3954 | <u>UBQ 10 Promoter</u> (underlined) |
| 3955 – 4746 | NPTII (highlighted blue) |
| 4747 – 5014 | NOS Terminator (bold) |
| 5155 – 5241 | 2 Left Borders (<i>italic</i>) |
| 2741 | XmnI Recognition Sequence (highlighted pink) |
| 3144 | EcoRI Recognition Sequence (highlighted orange) |

```

1      gatctgggga accctgtgggt tggcatgcac atacaatgg acgaacggat aaacctttc acgccctttt aaatatccga
81     ttattctaattaaacgctctt ttctcttagg tttaccggcc aatatatccct gtcaaacact gatagttaaa acttttaattt
161    aaggtaacctg cagaagcttag caagttaca gaagctcaa ttgcaggagc ccgtannnnn nnnnnactag tgggcagatc
241    agctttctc tctttatttt ttatttctt ttacaatagt aaacaattgg gattgaacat aaattttat acatctatata
321    acccccgaact ctatTTTAT taggctgaca ctaacgagtt ggatctcaat cacccttattt atttatttttct tcataatttt
401    ttcgtataaaa ctataaaggg atatTTAGGT acaaataaaaa aaaaaaaccctt agaaccttca agatgttgta gttgaacgc
481    tgtatcacgt gcattaatta gtctctatct atccatattt taggtcacgaa gagtggaccc cggaaataat tgcaattattt
561    atttaaaaaa ttatgtctaaa ttaagaacat ttatttatac attaatgcgt ttttattgtt aaattctaaa aatttagccta
641    cttaatataat tttaaaaaaaat aaaattatgt ttaatctaattt ttctgttgtt tataactaaaa ataatgattt ctgttgaaga
721    gaaaaaaagag acaaaaaaaaaaa aaataacatt taggaataact acaataaattt attggaccgt taaaatcagc ttacagctca
801    ctaaggTTTT gtcctcaagc caaataattt aggagtaaga ctatgaatat caggaatagt ttatTTATA aaagaattttg
881    tggTTgattt ttaatctagt aaagtaagag aaacttgcgtt ttttttttttcaaggatTTTtttggatTTTtttggatTTTtttggat
961    aaaagtgata cgtgttagatc taggaagagg cgTTgcctat ttaaaaaggc taacccttcca caagaatta aggtgcaaga
1041   gtttggTTgtg agctataagc tagTTTatcg tgaggagaat agtacgttcc gggccggccgc atgcttagcctt aggtccgc
1121   gcagcaacaa ccagtgcctt agacactctc catcaacaaa ctctagctga tcaatcctat ctaagcttat tacatagcaa
1201   gcatggggta ctccaaaacc ctagtagctg gcctgttgc aatgtgttta ctgtccgg ccgttggc caccgaccctt
1281   gaccctctcc aggacttctg tggccgcac ctcgacggca aggccggctc ggtgaacggg cacacgtgca agcccatgtc
1361   ggaggccggc gacgacttcc tcttctcgctc caagttggcc aaggccggca acacgtccac cccgaacggc tccggcgtga
1441   cggagctcga cgtggccgag tggccggta ccaacacgct ggggtgttcc atgaaccgcg tggactttgc tcccgagggc
1521   accaaccac cacacatcca cccgcgtgcc accgagatcg gcatcgat gaaaggtagt gttctcgatgg gaatcccttgg
1601   cagcctcgac tccggaaaca agctctactc gagggtgggtc cgccggcggag agacgttcc catccccacgg ggcctcatgc

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Appendix IX. Sequence of pTACF3 T-DNA in Darling 4

Appendix X. Letter of USFS support for blight-tolerant American chestnuts



United States
Department of
Agriculture

Forest
Service

Washington Office

1400 Independence Avenue, SW
Washington, D.C. 20250

File Code: 3400
Date: APR 11 2018

Mr. Richard P. Keigwin, Jr.
Director
U.S. Environmental Protection Agency
Office of Pesticide Programs
Division Mail Code 7501P
1200 Pennsylvania Avenue, N.W.
Washington, D.C. 20460

Dear Mr. Keigwin:

The U.S. Department of Agriculture's Forest Service continues to support the American Chestnut Foundation (TACF), the State University of New York (SUNY), and state agency partners in the development of blight-tolerant American chestnuts. The goal of returning this ecologically keystone species is of great importance to the public and the benefits to the public. It is a priority for a coalition of federal agencies, states, non-profits, universities, and private citizens with the common goal of getting this treasured tree back on the landscape.

The Forest Service, Natural Resources Conservation Service, and TACF have established a cooperative Memoranda of Understanding for research and management activities to promote the reintroduction of blight-tolerant American chestnut seedlings on public and private lands. To date, the Forest Service has provided land for research plantings of over 20,000 American chestnuts. Almost \$8 million dollars has already been invested by the Forest Service on American chestnut restoration, the majority of which has been allocated to the production of blight-tolerant trees. Our partners have invested additional funding to produce, deploy, and study blight-tolerant American chestnuts. These partners depend on the continuation of monitoring and maintenance of existing Forest Service plantings within its native range.

The success of restoration can be greatly accelerated using new technologies through transgenic and outcrossing of the American chestnut. We understand the transgenic (TG) American chestnut are undergoing environmental safety reviews by Animal and Plant Health Inspection Service's Biotechnology Regulatory Services, and the Food and Drug Administration's biotechnology consultation process for genetically engineered foods. Taken together, these reviews provide a high degree of confidence to the public that TG American chestnut does not present significant risk to human health or the environment.

We have an excellent opportunity through the work of the Forest Service, TACF and SUNY-ESF to bring this iconic tree back to its native range. We ask for your support in this worthy restoration effort.

Sincerely,

VICTORIA CHRISTIANSEN
Interim Chief



Caring for the Land and Serving People

Printed on Recycled Paper

