State University of New York College of Environmental Sciences and Forestry Petition (19-309-01p) for Determination of Nonregulated Status for Blight-Tolerant Darling 58 American Chestnut

OECD Unique Identifier:
ESF-DAR58-3

Draft Plant Pest Risk Assessment

June 2022

Agency Contact
Cindy Eck
Biotechnology Regulatory Services
4700 River Road
USDA, APHIS
Riverdale, MD 20737
Fax: (301) 734-8669

TABLE OF CONTENTS **PAGE**

A. Introduction

The State University of New York College of Environmental Science and Forestry (hereafter referred to as ESF) has submitted a petition to Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) seeking a determination of nonregulated status for American chestnut (*Castanea dentata*) event with OECD Unique Identifier "ESF-DAR58-3" (hereafter referred to as Darling 58 American chestnut) developed using genetic engineering (hereafter referred to as modified) for enhanced blight tolerance. The petition presents evidence supporting the argument that Darling 58 American chestnut is unlikely to pose a plant pest risk and, therefore, should no longer be regulated under the APHIS' 7 Code of Federal Regulations (CFR) part 340. This petition was assigned the number 19-309-01p and is hereafter referenced as ESF 2019 (ESF 2019). Under the authority of the plant pest provisions of the Plant Protection Act (7 U.S.C. 7701 *et seq.* 2019), the regulations in 7 CFR part 340, "Movement of Organisms Modified or Produced Through Genetic Engineering," regulate, among other things, the importation, interstate movement, and release into the environment of organisms modified or produced through genetic engineering that are plant pests or pose a plausible plant pest risk. This plant pest risk assessment (PPRA) was conducted to determine if the Darling 58 American chestnut is unlikely to pose a plant pest risk.

The petition for a determination of nonregulated status described in this PPRA is being evaluated under the version of the regulations effective at the time the petition was received. APHIS issued a final rule, published in the Federal Register on May 18, 2020 (85 FR 29790-29838, Docket No. APHIS-2018-0034)[1], revising 7 CFR part 340. Since the petition for determination of nonregulated status for Darling 58 American chestnut was received on January 21, 2020, before the revisions to the regulations became final, this petition request is being evaluated in accordance with the [legacy] regulations at 7 CFR 340.6 (e) (2020).

Darling 58 American chestnut was produced by *Agrobacterium tumefaciens*-mediated transformation of somatic embryos from the isogenic line of Darling 58 known as Ellis using plasmid p35S-OxO (ESF 2019). Portions of the inserted genetic material were derived from plant pest organisms listed in 7 CFR § 340.2 (7 CFR 340 2020) (i.e., a promoter sequence from cauliflower mosaic caulimovirus (CaMV) and a terminator sequence from *A. tumefaciens*) (Section 7.1, pp. 86-87, ESF 2019). Therefore, Darling 58 American chestnut is considered regulated under APHIS regulations at 7 CFR part 340 (7 CFR 340 2020). ESF has conducted field releases of Darling 58 American chestnut under APHIS authorizations since 2014 (ESF 2019), in part, to gather information to support that Darling 58 American Chestnut is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived.

Potential impacts in this plant pest risk assessment are those that pertain to plant pest risk associated with Darling 58 American chestnut and its progeny and their use in the absence of confinement relative to the unmodified recipient line and/or other appropriate

[1] To view the final rule, go to www.regulations.gov and enter APHIS-2018-0034 in the Search field.

comparators. APHIS utilizes data and information submitted by the applicant, in addition to current literature, to determine if Darling 58 American chestnut is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived. APHIS regulations in 7 CFR § 340.6(c) (7 CFR 340 2020) specify the information needed for consideration in a petition for nonregulated status. APHIS will assess information submitted by the applicant about Darling 58 American chestnut related to: plant pest risk characteristics; expression of the gene product, new enzymes, or changes to plant metabolism; disease and pest susceptibilities and indirect plant pest effects on other agricultural products; effects of the regulated article on nontarget organisms; weediness of the regulated article; impact on the weediness of any other plant with which it can interbreed; changes to agricultural or cultivation practices that may impact diseases and pests of plants; and transfer of genetic information to organisms with which it cannot interbreed.

APHIS may also consider information relevant to reviews conducted by other agencies that are part of the 'Coordinated Framework for the Regulation of Biotechnology' (51 FR 23302 1986; 57 FR 22984 1992) Under the Coordinated Framework, the oversight of biotechnology-derived plants rests with APHIS, the Food and Drug Administration (FDA), and the Office of Pesticide Programs of the U.S. Environmental Protection Agency (EPA). Depending on its characteristics, certain biotechnology-derived products are subjected to review by one or more of these agencies.

Under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136 *et seq.* 2019 Edition), EPA regulates the distribution, sale, use and testing of pesticidal substances produced in plants and microbes, including those pesticides produced by an organism through techniques of modern biotechnology. EPA also sets tolerance limits for residues of pesticides on and in food and animal feed, or establishes an exemption from the requirement for a tolerance, under the Federal Food, Drug and Cosmetic Act (FFDCA) (21 U.S.C. 301 *et seq.* 2018 Edition.). Prior to registration for a new use for a new or previously registered pesticide, EPA must determine through testing that the pesticide does not cause unreasonable adverse effects on humans, the environment, and nontarget species when used in accordance with label instructions. EPA must also approve the language used on the pesticide label in accordance with Data Requirements for Pesticides (40 CFR part 158 2019 Edition.). Other applicable EPA regulations include Pesticide Registration and Classification Procedures (40 CFR part 152 2019 Edition.), Procedures and Requirements for Plant Incorporated Protectants (PIPs) (40 CFR part 174 2019 Edition.), and Experimental Use Permits (40 CFR part 172 2019 Edition.). ESF informed APHIS that EPA has deemed the expressed OxO protein in their Darling 58 American chestnut tree as a PIP and therefore it must be registered as a pesticide under Section 3 of FIFRA. However, EPA also advised ESF that Darling 58 American chestnut may qualify for an exemption under Section 25(b) of FIFRA, so ESF has decided to go through both the Section 3 FIFRA registration and the Section 25(b) FIFRA exemption processes simultaneously. After EPA has completed its assessments of ESF's submissions and provided these to APHIS, APHIS will update this PPRA if needed.

The FDA under the FFDCA is responsible for ensuring the safety and proper labeling of all plant-derived foods and feeds, including those developed through modern

biotechnology. To help sponsors of foods and feeds derived from modified crops comply with their obligations, the FDA encourages them to participate in its voluntary early food safety evaluation for new non-pesticidal proteins produced by new plant varieties intended to be used as food (US-FDA 2006) and a more comprehensive voluntary consultation process prior to commercial distribution of food or feed (57 FR 22984 1992). ESF submitted a voluntary consultation with the FDA on October 28, 2021, concerning food and feed derived from Darling 58 American chestnut and is awaiting FDA's response.

B. Development of Blight-Tolerant Darling 58 American Chestnut

American chestnut (*Castanea dentata*) was once a dominant forest tree and keystone species within its native range that extended across eastern North America, including nearly every state east of the Mississippi as well as southern Ontario, Canada (Little 1977; Shaw et al. 2012; ESF 2019). Prior to the introduction of *Cryphonectria parasitica* to the United States, American chestnut seeds were primarily collected from wild trees for animal feed or human use by local farmers. Wild trees were also harvested for timber. Specimen trees were also planted around farmhouses, along residential streets, and in city parks, but due to the abundance of wild trees across forests in the Eastern United States, the wild long-lived trees provided ample nuts to meet demand for anthropocentric uses.

Once a major component of the Eastern hardwood forest and in some areas comprising half of the hardwood tree population, American chestnut is almost extinct in the wild now, having succumbed to chestnut blight, a bark fungal disease introduced from Asia caused by the fungus *C. parasitica*. The chestnut blight fungus kills the above-ground portion of American chestnut trees and as a result, the American chestnut now persists mostly in the form of sprouts from old stumps and root systems. Typically, the sprouts grow up and possibly flower and fruit for several years before dying back from the blight. Before blight introduction, mature trees could reach more than 35 meters tall (>120 feet) and 5 meters wide (>17 feet) (Davis 2005). American chestnut is now considered functionally extint in modern forests (ESF 2019).

To rescue and ultimately restore American chestnut tree in it native environment, the petitioner has developed Darling 58 American chestnut trees with enhanced tolerance to chestnut blight. Darling 58 American chestnut was developed from a single immature zygotic embryo extracted from a wild-type American chestnut seed. The embryo was first subcultured to produce somatic embryo, and then the somatic embryo clumps were transformed with disarmed *A. tumefaciens* strain AGL1 containing the plasmid vector p35S-OxO (Figure 7.1a, p. 85, ESF 2019). The p35S-OxO *Agrobacterium* vector contains the *oxo* and *nptII* expression cassettes that confer tolerance to chestnut blight and resistance to aminoglycoside antibiotics, respectively, and plasmid backbone sequences necessary for maintenance or selection of the plasmid vector in bacteria but which are not expected to be transferred to the American chestnut somatic embryo (Figure 7.1a., p. 85, ESF 2019).

After incubation with the *Agrobacterium* vector, the American chestnut somatic embryo clumps were placed on selection medium with hygromycin to select for transformed embryos and with the antibiotic carbenicillin disodium salt to eliminate *A. tumefaciens*. Each clump of surviving somatic embryo tissue on selection medium was considered a separate event, presumably originating from a single transformed cell. Putative transgenic somatic embryos (events) were propagated and then tested for the presence of *oxo* gene via PCR screening. The somatic embryos with the confirmed presence of *oxo* gene were propagated and regenerated. The regenerated shoot was then placed on rooting medium to induce the development of roots, resulting in the production of transformed American chestnut plantlets (ESF 2019).

The transformed American chestnut plant (Darling 58) expresses the *oxo* gene encoding oxalate oxidase (OxO) that neutralizes oxalic acid produced by the chestnut blight fungus (ESF 2019). Additionally, Darling 58 contains selectable marker gene *nptII* that expresses the neomycin phosphotransferase (NPTII) enzyme to allow successfully transformed tissue to survive in the presence of aminoglycoside antibiotics. The NPTII protein is produced in numerous deregulated commercial plant products, and the safety of NPTII proteins present in biotechnology-derived crops has been extensively assessed (US-FDA 2022; USDA-APHIS 2022).

The isogenic recipient genome of Darling 58 is a wild-type *C. dentata* tissue culture line known as Ellis that was established from a single immature zygotic embryo extracted from wild seed of an American chestnut tree (*C. dentata* (Marsh.) Borkh.) known as Pond #1 (ESF 2019). This tree is located near Windsor, NY, on the property of a member of The American Chestnut Foundation who reports that in the early 1960s, the property contained hundreds of naturalized American chestnuts, including the Pond #1 tree. 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.

Control materials used as comparators in the Darling 58 safety assessments include the isogenic recipient line Ellis and unmodified full siblings for molecular characterization studies (vector insert copy number, insert location, OxO mRNA expression, OxO enzyme activity, OxO enzyme quantification). Control materials used as comparators for phenotypic characteristics (blight tolerance, growth, respiration and photosynthesis, nut nutrition and composition) and environmental interactions (mycorrhizal colonization of roots, seed germination in Darling 58 leaf litter, insect herbivory on Darling 58 leaves, bumble bee foraging on Darling 58 pollen, and responses to other pests and diseases) included wild-type American chestnut, full-siblings, traditionally bred American chestnut x Chinese Chestnut hybrids, Chinese chestnut, and European chestnut (ESF 2019). APHIS is confident that the use of the unmodified isogenic recipient line, unmodified full siblings, and the other reference materials used as comparators is sufficient to determine that Darling 58 American chestnut does not differ from other American chestnut growing wild or in an orchard.

C. Description of Inserted Genetic Material, Its Inheritance and Expression, Gene Products, and Changes to Plant Metabolism

To inform the potential hazards resulting from the genetic modification and potential routes of exposure related to the inserted DNA and its expression products, APHIS assessed data and information presented in the petition related to the transformation process; the source of the inserted genetic material and its function in both the donor organism and the modified crop event; and the integrity, stability, and mode of inheritance of the inserted genetic material through sexual or asexual reproduction based on the location of the insertion (e.g., nucleus or organelle) and the number of loci inserted.

APHIS also assessed data presented in the petition on whether the genetic modification results in expression of new genes, proteins, or enzymes, or changes in plant metabolism or composition in Darling 58 American chestnut compared to the isogenic and other non-transgenic controls. The assessment encompasses a consideration of the expressed oxalate oxidase (OxO) from *Triticum aestivum*, the expressed neomycyin phosphotransferase (NPTII) from *Escherichia coli*, and any observed or anticipated effects on plant metabolism including, for example, any relevant changes in levels of metabolites, antinutrients, or nutrients in nuts or leaves derived from Darling 58 American chestnut compared to those in the isogenic or other non-transgenic controls.

This information is used later in this risk assessment to inform whether there is any potential for plant pest vectors or sequences to cause disease or greater plant pest risks in the modified chestnut tree event; or for expression of inserted DNA, new proteins or enzymes, or changes in metabolism to affect plant pests or diseases, nontarget beneficial organisms, weediness, silvicultural and agricultural practices that impact pests or diseases or their management, or plant pest risks through horizontal gene flow.

Description of the genetic modification and inheritance of inserted DNA

The inserted DNA in Darling58 American chestnut, with both the *oxo* and *nptII* gene expression cassettes from T-DNA for production of OxO and NPTII proteins, is described as containing the following genetic elements (Figure 7.1a, p85, ESF 2019):

- **B-Right Border Region**: A specific DNA region from *A. tumefaciens* containing the 71 base pair right border sequence used for transfer of the T-DNA.
- ***CaMV*: Promoter Sequence** of the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Guilley et al. 1982).
- **Ta-*oxo*: Coding Sequence,** 672 base pairs, for oxalate oxidase enzyme (OxO) protein from *Triticum aestivum* that confers tolerance to blight in American chestnut.
- **T-*actII*: Terminator Sequence**, actin 2 terminator from *Arabidopsis thaliana*.
- **P-*UBQ10*: Promoter Sequence**, the constitutive promoter of the Ubiquitin 10 gene from *Arabidopsis thaliana*.

- **CS-*nptII*: Coding Sequence**, for neomycyin phosphotransferase (NPTII) from *Escherichia coli*.
- **T*nos*: Terminator Sequence**, 3' UTR from nopaline synthase gene, *nos*, of *A. tumefaciens*.
- **B-Left Border Region:** A specific DNA region from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA.

ESF confirmed the insertion of the genetic elements listed above by conducting a detailed molecular characterization of the inserted DNA sequences in Darling 58 American chestnut tree. ESF used a combination of PCR and Sequence Analysis to demonstrate the insertion of transgenes (*oxo* and *nptII* genes) but without causing any extraneous insertion of vector DNA sequences or deletions of existing DNA compared to the Ellis isogenic line. APHIS reviewed the molecular characterization data and methods provided in Section 7 of the petition (ESF 2019):

- The T-DNA inserted into the Darling 58 American chestnut genome is present at a single locus on chromosome 7, about 10.9 and 5.5 kilobases away from the nearest upstream and downstream chestnut genes, respectively.
- Darling 58 does not contain any sequence from the plasmid p35S-OxO backbone or from T-DNA.
- The T-DNA sequence in Darling 58 has an inversion of about 600 base pairs of genomic DNA just outside the left T-DNA border. However, the inversion is not near any known genes.

Expression of inserted DNA, changes in gene expression, new proteins or metabolism

Blight tolerance in Darling 58 American chestnut is conferred by an oxalate oxidase gene (*oxo*) from *T. aestivum*. The mechanism of the blight tolerance mediated by oxalate oxidase enzyme is to detoxify oxalic acid secreted by the fungal pathogen, preventing the oxalate acid from killing the chestnut's tissues. Oxalate oxidase is widely present in all grains of crops and many wild plants and microbes, and it is well understood with a history of over 100 years' studies. Wheat oxalate oxidase enzyme has been well characterized, can effectively detoxify oxalate, and has no known safety concern for consumption by human and livestock.

The Darling 58 oxalate oxidase enzyme was shown to be expressed in all the tested leaf, stem, root, and nut tissues of the chestnut tree, but in a decremental sequential order.

The petitioner carried out nut nutrition and composition analysis of Darling 58 and its non-transgenic American chestnuts to assess whether levels of key nutrients and compositions in Darling 58 American chestnut were equivalent to levels in the non-transgenic control. It showed that Darling 58 and non-transgenic nuts are nutritionally almost identical when rounded to FDA guidelines for food labels. While difference does exist in American chestnut nutritional measurements between source trees, and between similar trees over different years, the values of transgenic chestnuts are within the ranges

found in non-transgenic chestnuts. This supports the conclusion that Darling 58 American chestnut is both nutritionally and compositionally similar to its related non-transgenic controls.

Based on all the above noted considerations, APHIS concludes that the inserted genetic material, its inheritance and expression are stable, and the resulting proteins do not alter Americann chestnut plant metabolisms except the intended blight tolerance trait compared to non-transgenic controls.

D. Potential Plant Pest and Disease Impacts

APHIS assessed whether potential plant pest or disease impacts are likely to result from the transformation process, from DNA sequences from plant pests, or from any other expression products, new enzymes, proteins or changes in plant metabolism or composition in Darling 58 American chestnut that are known or anticipated to cause disease symptoms, or to affect plant pests or diseases or plant defense responses (as identified from the previous section). APHIS also assessed whether Darling 58 American chestnut is likely to have significantly increased disease and pest susceptibility based on data and observations from field trials on specific pest and disease damage or incidence and any agronomic data that might relate to such damage. Impacts or changes are assessed to determine if they would (1) affect the new modified crop and/or result in significant introduction or spread of a damaging pest or disease to other plants; (2) result in the introduction, spread, and/or creation of a new disease; and/or (3) result in a significant exacerbation of a pest or disease for which APHIS has a control program. Any increase in pest or disease susceptibility is evaluated with respect to the context of currently cultivated varieties, the ability to manage the pest or disease, and the potential impact on agriculture.

American chestnut itself is not considered a plant pest in the U.S. (7 CFR § 340.2 (7 CFR 340 2020)). The plant pest derived vector DNA and the plant pest vector used to insert the DNA do not pose a plant pest risk to Darling 58 American chestnut. The binary plasmid vector p35S-OxO proved to be disarmed; the T-DNA inserted into Darling 58 American chestnut contained only the intended sequences, along with the typical insertion site mutations, and lacked sequences from Tumor-inducing (Ti) plasmids normally responsible for the formation of crown gall tumors upon *A. tumefaciens* infection (Hoekema et al. 1983; Hellens et al. 2000). The sequences derived from plant pathogens retained in Darling 58 American chestnut (i.e., promoter sequence from caulimovirus and T-DNA border sequences from *A. tumefaciens*) are non-coding sequences that do not cause plant disease.

Darling 58 American chestnut was grown within confined field trials in the U.S. since 2011 across seven states covering a diverse range of environmental conditions representative of where American chestnut is currently grown and bred, and where Darling 58 American chestnut is expected to be grown. In addition to the observational data that ESF annually reported to USDA-APHIS from these product development trials,

which would have included reports of unusual pest and/or disease incidence, ESF also assessed phenotypic and environmental interaction characteristics for Darling 58 American chestnut compared with the non-transgenic controls.

Data from these tests showed that the most substantial difference in Darling 58 American chestnuts compared to the non-transgenic controls is its oxalic acid tolerance that allows the tree to coexist with the blight fungus. However, Darling 58 American chestnuts are not associated with increased susceptibility or tolerance to specific abiotic stressors, arthropods, or diseases compared to controls. The results support the conclusion that the blight tolerance trait in Darling 58 American chestnut is not expected to alter the response of Darling 58 to diseases or arthropod pests under natural levels of these stressors, nor cause pest arthropods to be more abundant around Darling 58 plots, compared to conventional non-transgenic controls.

In summary, the introduced genes did not significantly alter the observed insect pest infestation and disease occurrence or resulting damage on Darling 58 compared to the control and other reference lines. As presented later in this document, the blight tolerance trait did not significantly alter the observed agronomic and phenotypic traits and did not reveal any significant changes that would indirectly indicate that Darling 58 is or could be more susceptible to pests and diseases compared to the control lines. Thus, Darling 58 is unlikely to be more susceptible to plant pathogens and insect pests than conventional American chestnut and existing commercial hybrids, and it is unlikely to differ from conventional American chestnut in its ability to harbor or transmit plant pathogens or pests and cause indirect plant pest effects on other agricultural products.

E. Potential Impacts on Nontarget Organisms Beneficial to Agriculture

APHIS assessed whether exposure or consumption of Darling 58 American chestnut would have a direct or indirect adverse impact on species beneficial to agriculture. Organisms considered were representatives of the species associated with production of the regulated crop in the agricultural environment. The assessment includes an analysis of data and information on Darling 58 American chestnut compared to the conventional control and other comparators used as a reference range for 1) any biologically relevant changes in the phenotype or substances produced that may be novel or expressed at significantly altered amounts and are associated with impacts on organisms beneficial to agriculture, and/or 2) any observations of beneficial organisms associated with the plants. APHIS reviewed information ESF provided justifying the safety of Darling 58, as well as additional literature.

As indicated earlier in this plant pest risk assessment, the mechanism of the blight defense in Darling 58 American chestnut is mediated by wheat oxalate oxidase enzyme to detoxify oxalic acid secreted by the fungal pathogen, which allows the tree to tolerate and coexist with the blight fungus. Also, oxalate oxidase is widely present in a variety of plants and microbes, has been well characterized, and has no known safety concern for consumption by human and livestock. The petitioner's characterization of Darling 58

showed nutrient composition levels in nuts were comparable to the conventional control, and that the Darling 58 OxO expression level is low, so there is unlikely to be nontarget effects resulting from changes in composition or from consumption of Darling 58 OxO. Also, the study on environmental interactions found that there were no changes in beneficial arthropod abundance in field plots of Darling 58.

Therefore, based on the above analysis of ESF's studies on Darling 58 nut nutrient and composition, levels of OxO in tissues, and environmental interactions with beneficial arthropods, APHIS concludes exposure to and/or consumption of Darling 58 are unlikely to have any adverse impacts on organisms beneficial to agriculture.

F. Potential for Enhanced Weediness of Darling 58 American Chestnut

APHIS assessed whether Darling 58 is likely to become more weedy (i.e., more prevalent, competitive, damaging or difficult-to-control in situations where it is not wanted) than the non-transgenic controls. The assessment considers the basic biology of the plant, the situations in which volunteers or feral populations are considered weeds, and an evaluation of Darling 58 compared to its progenitor chestnuts under field conditions characteristic for the regions of the U.S. where American chestnut is grown (and/or evaluated under laboratory or greenhouse conditions) for characteristics related to establishment, competiveness, reproduction, survival, persistence and/or spread that could influence weediness and the ability to manage the crop as a weed. The assessment also considers whether the engineered trait affects methods of control for the plant in situations where it is managed as a weed or volunteer in subsequent crops or in feral populations.

American chestnut is a generalist species with competitive growth rate, it exhibits intermediate shade tolerance, and ability to regenerate vegetatively from the root collar (Jacobs et al. 2013). These traits allow American chestnut to establish and colonize areas where soil and climatic conditions are suitable, which includes much of the eastern United States and southeastern Canada. Though American chestnut grows considerably faster than other hardwoods on the same site (McEwan et al. 2006), American chestnut spreads at an average rate of 100 m/yr., which is much slower than oaks which spread at an average rate of 350 m/yr., and American beech at an average rate of 200 m/yr. (Davis 1981; Davis 1983; McEwan et al. 2006). This slow-spread of American chestnut trees may be due to the production of 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; ESF 2019).

The natural spread of chestnut species to a new location can occur only by seed because chestnut sprouts new shoots only from the immediate location of the former tree (ESF 2019). However, American chestnut takes at least several years to produce seeds, and a good number of seeds would be produced only after 20th year (Zon 1904; Cook and Forest 1979). American chestnut seeds show little dormancy because mature seeds are released from burs through fall frost and then can sprout the following spring (ESF

2019). Furthermore, actual seed germination and the subsequent seedling establishment of chestnut is rare due to the chestnut seeds' high predation rate by wildlife and insects. Even after being established, chestnut seedlings are also subject to herbivory and damage by a variety of mammals. Therefore, American chestnut trees are not able to spread quickly without human assistance.

American chestnut is better adapted to fire and intensive logging than its competitors due to its ability to sprout after fire or from the cut stumps (Abrams 2003; Nowacki and Abrams 2008; Faison and Foster 2014). It was also empirically shown that American chestnut has the ecological capacity to achieve canopy dominance on favorable sites (Paillet and Rutter 1989). One of the most reliable predictors of whether a species would be invasive was whether the plant was known to be invasive elsewhere in the world (Reichard and Hamilton 1997). Chestnuts have been introduced throughout the United States, as well as in Argentina, Australia, and New Zealand (Jaynes and DePalma 1984). However, American chestnut has become naturalized in only a few places outside its native range (Section 2.1, ESF 2019). 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, ESF 2019). These data indicate that Darling 58 American chestnut is unlikely to display the highly competitive and fast spreading behavior associated with weedy and/or invasive species.

In the United States, American chestnut is not listed as a weed in any of the major weed databases, nor is it designated as a noxious weed or invasive species by the United States federal government or any other countries. American chestnut exhibited 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. Considering that there are no differences in the seedling establishment, growth, reproduction and dispersal of Darling 58 and wild-type American chestnut, it is implied that Darling 58 American chestnut trees may also take a century or more to become dominant after the first pioneer trees become established in a given area and it is unlikely that Darling 58 American chestnut populations establish in non-native habitats.

G. Potential Impacts on the Weediness of Any Other Plants with which Darling 58 American Chestnut Can Interbreed

Gene flow is a natural biological process with significant evolutionary importance. A number of angiosperm taxa are believed to be derived from hybridization or introgression between closely related taxa (Grant 1981; Rieseberg and Wendel 1993; Soltis et al. 1993; Grant 1994; Hegde et al. 2006) and even in the existing floras, the occurrence of hybridization or introgression is reported to be widespread (Stace 1987; Rieseberg and Wendel 1993; Rieseberg 1997; Preston et al. 2002). It has been a common practice by plant breeders to artificially introgress traits from wild relatives into crop plants to develop new cultivars (Khoury et al. 2013). However, gene flow from crops to wild relatives is also thought of as having a potential to enhance the weediness of wild

relatives, as observed in rice, sorghum, sunflower and a few other crops (see Table 1 in Ellstrand et al. (1999)). By providing fitness-related traits such as resistance to insects, diseases, herbicides or harsh growing conditions, gene flow from crops to their wild relatives could allow the hybrids to compete better, produce more seeds, and become more abundant (Snow 2002). Besides weediness, other concerns are the loss of herbicide resistance as a tool to protect crops from closely related weeds (Gepts and Papa 2003). This topic is covered in two sections: 1) the potential for gene flow, hybridization and introgression from Darling 58 to sexually compatible relatives, including wild, weedy, feral or cultivated species in the United States and its territories, and 2) if so, the risk potential with respect to weediness of those taxa following introgression, based on the phenotypic changes that have been observed in the engineered plants.

Potential for gene flow, hybridization and gene introgression

The American chestnut (*Castanea dentata* (Marsh.) Borkh) is a deciduous tree with a widespread historical range in the eastern United States and southeastern Canada before the introduced blight killed the chestnut trees in the early 20th century (Emily 1987). Fossils of Castanea that were detected in Greenland and throughout western North America and Asia can be dated back to more than 85-60 million years ago (mya) and indicate that the species once had a much wider distribution than today (Dane 2022). There are at least 26 *Castanea* species known to occur in North America (GBIF 2022) of which the occurance of some *Castanea* species (*C. dentata*, *C. crenata*, *C. mill*, *C x neglecta*, *C. pumila*, *C. sativa*) were also mapped by USDA and found substantial overlap in their range with American chestnut (USDA-NRCS 2022). Also, figure 2.1.1a of this petition describes overlap of distribution range for North American Castanea species: *C. dentata*, *C. pumila*, and *C. ozarkensis*. However, none of the *Castanea* species are listed as weeds or invasive species (CABI 2022; WSSA 2022).

C. dentata (American chestnut) is substantially outcrossing and self-incompatible. A recent whole genome analysis of 384 putative American chestnut individuals showed that 340 had >99% ancestry assigned to the *C. dentata* populations. Whereas ten individuals showed a significant level of ancestry (>10%) from other *Castanea* species. Of these four samples were identified as *C. sativa* x *C. dentata* hybrids, and three were *C. mollissima* x *C. dentata* hybrids. Overall, the American chestnut samples sequenced revealed some degree of introgression with other *Castanea* species (Sandercock et al. 2022). It was also described in the petition that the 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 and DePalma 1984) to form hybrids (Section 2.1.2). However, many such crosses may result in problems such as abnormal nut development called Internal Kernel Breakdown (Fulbright et al. 2012), a semi-lethal condition called “cracked bark” in young seedlings (Jaynes and DePalma 1984), and male sterility in hybrid offspring (Anagnostakis 2012; Sisco et al. 2012).

The widespread chestnut blight fungus, which colonizes wounded cambium and forms a canker that eventually results in death of the aboveground stem, has led to the decline of sexual reproduction of American chestnut in the North American range to the point of being very rare. The blight does not attack roots, so the current remaining wild American

chestnut population is characterized by an asexual cycle of root-collar sprouting from the surviving trunks, eventual infection by blight before the tree can reach the forest canopy and form flowers, and stem dieback. In the U.S., 431 ± 30.2 million American chestnut trunks remain with the vast majority of these trunks (360 ± 22 million, 84%) having a diameter at breast height (dbh) of <2.5 cm. (Dalgleish et al. 2015). The pollen-flow from Darling 58 to these American chestnut trunks and to the sprouts emerging from them, will not result in the successful production of hybrid offspring.

There are several reports of effective pollination distance for American chestnut from reviewed and non-reviewed sources. These reports would include both wind and insect pollination because they examine viable nut production success, regardless of how the pollen was vectored. Jacobs et al. (2016) report that trees need to be within 100 meters (~ 328 ft) for successful pollination. Rutter (1990) states that trees only 100 feet apart have reduced pollination success, and trees 1000 ft apart are essentially reproductively isolated from one another. 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 1000 ft from each other to prevent pollination (Long 2012).

Pollen-mediated gene-flow from events derived from Darling 58 to the wild relatives would be possible because *Castaenea* species can hybridize freely (Lang et al. 2006). Also, there are reports of natural occurance of interspecific hybrids such as *C. dentata* x *C. mollissima* and *Castenea* x *neglecta* (a natural hybrid between *C. dentata* x *C. pumila*) with *C. dentata* as one of the parental species (Binkley 2008; Perkins et al. 2019; Sandercock et al. 2022). These analyses suggest that there is a low degree of possibility for gene flow via introgression between sympatric Darling 58 American chestnut and other *Castanea* species.

Potential for enhanced weediness of recipients after hybridization and/or introgression

Darling 58 American chestnut, described in the current petition, was transformed with *oxo* gene to produce oxalate oxidase (OxO) enzyme that can detoxify the oxalate produced by the blight-causing fungus, *Cryphonectria parasitica*. The OxO enzyme detoxify the oxalate by breaking it down to carbon dioxide and hydrogen peroxide. The hydrogen peroxide byproduct might have a second function of strengthening the lignin in the barrier produced by the chestnut in an effort to wall off the fungal infection. Though there is some evidence that the OxO enzyme can enhance lignin formation (Welch et al. 2007), this is not to modify any other traits related to weediness such as competitiveness, responses to other pests, interactions with other organisms in the environment, or survival (besides blight tolerance).

If genetic introgression of the transgene in Darling 58 American chestnut to a sexually compatible relative were to occur, the offspring would be expected to gain the ability to synthesize oxalate oxidase and tolerance to blight fungus. However, similarly, to modified chestnut, the OxO production is not expected to influence the weediness of sexually compatible relatives of chestnut that acquire the transgene. American chestnut

and its wild relative are not listed as weeds or invasive species by any US states or other countries.

Based on the information presented in the petition and in relevant literature, APHIS has reached the following conclusions. Gene flow, hybridization and/or introgression of genes from American chestnut to its sexually compatible relatives, including wild, feral or cultivated species in the U.S. and its territories is likely to occur if the plants are within the pollen dispersal distance, and depending on their potential for crossing with *C. dentata*, as discussed above. The genetic modification in Darling 58 American chestnut is not expected to increase the potential for hybridization, gene flow and and/or introgression to occur to sexually compatible taxa compared to the non-transgenic recipient or other varieties of chestnut that are commonly growing in the forests and in the sylviculture settings. The genetic modification in Darling 58 American chestnut is unlikely to confer novel weedy properties to chestnut or its wild relatives. Therefore, APHIS has determined that Darling 58 American chestnut is not expected to increase the weed risk potential of other species with which it can interbreed in the U.S. and its territories.

H. Potential Changes to Silviculture or Agriculture Practices

APHIS assessed whether significant changes to silvicultural or agricultural practices from adoption of Darling 58 are likely to impact American chestnut's silviculture or agriculture practices. This includes consideration of any changes in phenotypic characteristics and environmental interactions.

The American chestnut was once one of the most abundant trees within its native range in the eastern United States. It was a fast-growing and long-lived canopy tree that produced a consistent crop of nuts, could be harvested for lumber, and was considered a keystone species for wildlife in the past. However, American chestnuts are now considered functionally extinct due to a fungal canker disease caused by an invasive fungal pathogen *C. parasitica*.

ESF intends to use Darling 58 blight-tolerant American chestnut for restoration and forest health and to establish and colonize much of the eastern United States where stunted trees persist. The long-term goal is for the American chestnut to become a self-sustaining forest tree species within its native range. Initial distribution will consist of long-term research plots and relatively small-scale public horticultural plantings to inform subsequent larger-scale distributions. The American Chestnut Foundation (TACF) and ESF 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.

As previously described, tolerance to the invasive, exotic fungal pathogen, *C. parasitica*, in Darling 58 American chestnuts was enhanced by inserting the wheat *oxo* gene for the oxalate oxidase (OxO) enzyme. 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. And thus, the introduced oxalic acid tolerance conferred by *oxo* gene allows coexistence of American chestnut tree and the fungus. This means that modified American chestnut trees will not require forest management intervention practices used to maintain a plant defense mechanism.

Control materials used as comparators for phenotypic characteristics (blight tolerance, growth, respiration and photosynthesis) and environmental interactions (mycorrhizal colonization of roots, seed germination in Darling 58 leaf litter, insect herbivory on Darling 58 leaves, bumble bee foraging on Darling 58 pollen, and responses to other pests and diseases) included wild-type American chestnut, full-siblings, traditionally bred American chestnut x Chinese Chestnut hybrids, Chinese chestnut, and European chestnut (ESF 2019).

Phenotypic observations including growth and photosynthesis (Section 8) and including growth and photosynthesis and environmental interaction experiments on Darling 58 have been conducted under laboratories, greenhouses, and field conditions under APHIS BRS authorizations. The purpose of the authorizations includes *Phytophthora* screenings, outdoor plantings to evaluate growth, controlled pollinations, ecological interactions, and blight tolerance. No unusual occurrences, non-target interactions, or deleterious effects have been observed which demonstrate that Darling 58 is not significantly different from unmodified chestnut controls (Appendix I, ESF 2019).

Phenotypic characteristics

ESF evaluated phenotypic characteristics as compared to unmodified and traditionally bred controls in field, laboratory, or greenhouse experiments. Additionally, data from legacy modified event, Darling 4, provide further evidence to support that the presence of *oxo* gene or the transformation process does not significantly affect growth or performance of modified American chestnuts. Modified American chestnut trees had similar growth and photosynthetic and respiratory rates compared to unmodified control trees.

Furthermore, the data support the conclusion that the Darling 58 American 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/blight tolerance trait that allows chestnut trees to coexist with the blight fungus.

Environmental Interactions

Mycorrhizal colonization of Darling 58 roots
There were no significant differences in colonization by ectomycorrhizal fungi in roots compared to unmodified controls. 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 (ESF 2019). The introduction of Darling 58 back into eastern forest is unlikely to have negative impacts on soil quality.

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

A greenhouse bioassay was performed to evaluate interactions between leaf litter from modified American chestnut trees and seeds from other species that are native or naturalized in American chestnut habitats (Newhouse et al. 2018). No significant differences in seed germination in the presence of modified vs. unmodified American chestnut leaf litter, in terms of numbers of seeds germinated or total dry biomass of germinated seedlings was observed. These studies reinforce that modified American chestnuts are not substantially different than unmodified American chestnuts (apart from their enhanced blight tolerance), and do not pose a novel pest risk to several types of representative native plants.

Insect herbivory on chestnut leaves

Interactions between chestnuts and herbivorous insects tested to date are not significantly affected by OxO. Plant/herbivore/biocontrol interaction tests to evaluate gypsy moth (*Lymantria dispar*) caterpillars feeding on various modified chestnut lines confirmed that insect herbivory on OxO-expressing modified American chestnuts is not significantly different than that on unmodified controls. Darling 58 modified leaves do not differentially affect biopesticides or parasitoids used to control insect pests.

Bumble bees and pollen with OxO

As previously mentioned, oxalate oxidase itself does not have known harmful effects against living organisms, but the hydrogen peroxide byproduct can have antimicrobial properties (Baldry 1983) or sub-lethal effects on insects (Ramputh et al. 2002). Chestnut pollen has been shown to be especially nutritious to bumble bees (*Bombus terrestris*) (Tasei and Aupinel 2008). However, a realistic concentration of oxalate oxidase in chestnut pollen does not present novel risks to bumble bees (Section 9.1.4, ESF 2019).

Responses to other pests and environmental stresses

Apart from chestnut blight tolerance, no differences between modified and unmodified trees have been observed in response to plant-disease, plant-arthropod, or plant-environment interactions. Modified and unmodified American chestnuts in greenhouse conditions do not appear differentially susceptible to damage from common greenhouse pests such as mealybugs, spider mites, and powdery mildew.

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). Studies by ECF confirm 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.

Field-grown chestnuts have also been observed to sustain incidental insect herbivory, powdery mildew infections, and rodent chewing, regardless of transgene presence. There is no evidence that planting of Darling 58 American chestnuts would affect incidence of other pests or pathogens any more than planting traditionally bred chestnuts.

Potential spread of OxO to other *Castanea* species
There is no data on natural pollination rates for Darling 58 trees yet. However, there is no reason to expect that pollen viability, fertilization rates, or any other aspect of sexual reproduction would differ between Darling 58 American chestnut and unmodified American chestnut

Persistence of OxO activity in Darling 58 leaves
To address a potential concern over persistence of OxO activity in modified American chestnut leaves, ESF tested and monitored the OxO activity in mature leaves of late-season Darling 58 plants. It was shown that OxO activity in abscised leaves was detectable for over five days but was not detectable when a leaf dried enough to begin to curl (Section 9, ESF 2019). Furthermore, the similarity of results between modified lines with different expression levels suggests that overall OxO transcript levels in active plants do not substantially affect the persistence of OxO activity in desiccating/inactive tissues.

Potential effects of blight-tolerant chestnuts on *C. parasitica*
Darling 58 blight-tolerant trees can survive and serve as a host of *C. parasitica* indefinitely, allowing the fungus to persist, while blight-susceptible trees eventually die back.

Furthermore, reproductively mature Darling 58 trees will continue to produce unmodified, blight-susceptible offspring through crossing with other American chestnut trees for the following reasons. American chestnut normally needs two trees to produce viable nuts because it is monoecious and predominantly self-infertile. Darling 58 is a hemizygous event with only one copy of the inserted *oxo* gene present in the genome. Thus, a cross with such hemizygous transgenic tree as a parent will always result in the offspring without transgene. Even when two Darling 58 trees are crossed, about 25% of their offspring would be non-transgenic and blight-susceptible (Section 6.4, ESF 2019). 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.

Taken all together, Darling 58 American chestnut is similar to unmodified controls in its agronomic, phenotypic, environmental response and compositional characteristics and has levels of pests and diseases or their damage levels comparable to unmodified American chestnuts. Furthermore, Darling 58 trees will be reintroduced as a self-sustaining tree species in its native range and these trees will not require additional forest management interventions. Also, because the progeny of Darling 58 trees will include both plants with transgene and plants without transgene, the original wild-type American chestnut will be conserved in the future (ESF 2019). In conclusion, APHIS could not

identify any significant changes to silvicultural or agricultural or practices from adoption of the modified chestnut.

I. Potential Impacts from Transfer of Genetic Information to Organisms with which Darling 58 American Chestnut Cannot Interbreed

APHIS examined the potential for the new genetic material inserted into Darling 58 American chestnut to be horizontally transferred to other organisms without sexual reproduction and whether such an event could lead directly or indirectly to disease, damage, injury, or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

The horizontal gene transfer (HGT) between unrelated organisms is one of the most intensively studied fields in the biosciences since the 1940s (Soucy et al. 2015), and the issue gained extra attention with the release of modified plants into the environment (Dröge et al. 1998). Potential risks from stable HGT from genetically engineered organisms to another organism without reproduction or human intervention have been reviewed (Keese 2008b). Mechanisms of HGT include conjugation, transformation, and transduction, and other diverse mechanisms of DNA and RNA uptake and recombination and rearrangement, most notably through viruses and mobile genetic elements (Keese 2008b; Soucy et al. 2015). HGT has been a major contributor to the spread of antibiotic resistance amongst pathogenic bacteria; emergence of increased virulence in bacteria, eukaryotes and viruses; and, in the long run, to major transitions in evolution (Brown 2003b; Keeling and Palmer 2008; Keese 2008b).

Potential for horizontal gene transfer to bacteria, fungi, or invertebrates

Darling 58 American chestnut contains protein coding regions derived from the *oxo* gene from the wheat plant (*Triticum aestivum*) and the *nptII* gene from *Escherichia coli*. It also contains non-protein-coding regions from *A. tumefaciens*, *A. thaliana*, and the cauliflower mosaic virus (CaMV).

One example of HGT involves a class of enzymes similar to mono-oxygenase gene (DMO). Chakraborty et al. (2012) propose that HGT contributed to the distribution of ring-hydroxylating oxygenase (*rho*) genes among prokaryotic phyla (proteobacteria, actinobacteria, cyanobacteria, and archaea), and note that homologues of *rho* genes are found in plants (in strains of *Arabidopsis, Zea mays, Oryza sativa, Physcomitrella patens and Amaranthus tricolor*). Ring-hydroxylating oxygenases (RHO) catalyze the addition of hydroxyl groups to aromatic ring compounds, initiating one of the major pathways for oxidative degradation of both natural and synthetic aromatic compounds in the environment (Peng et al. 2010a, b). Dicamba mono-oxygenase is a unique type of RHO that initiates the degradation of dicamba by oxygenating the exocyclic methyl group, rather than the more conventional oxygenation of the aromatic ring of the substrate seen in most other RHOs (Dumitru et al. 2009). Chakraborty et al. (2012) suggest that

distribution and diversification of *rho* genes can be explained by the mechanisms of gene duplication, transposition events and DNA rearrangements in most cases.

In other cases, HGT is assumed to be the primary mechanism where occurrence of the genes was found to be limited to just one or two organisms within phyla (such as *rho* genes in some cyanobacteria, firmicutes and crenarchaeota), since the possibility of being remnants of a partially deleted *rho* operon is ruled out due to the absence of similar genes in any other member of these genera. Although it is widely accepted that HGT has generated novel degradation capabilities and increased metabolic diversity among bacterial communities exposed to an ever-evolving array of polycyclic aromatic compounds, such degradative capabilities are mostly indicative of divergent evolution from a common ancestor, not HGT (Peng et al. 2010a).

Horizontal gene transfer and expression of DNA from a plant species to bacterial, fungal, or invertebrate species is unlikely to occur based on the following observations. Although there are many opportunities for plants to directly interact with fungi and bacteria (e.g., as commensals, symbionts, parasites, pathogens, decomposers, or in the guts of herbivores) and with invertebrates as plant pests, there are almost no evolutionary examples of HGT from eukaryotes to bacteria or from plants to fungi or invertebrates (Keese 2008b). Examples of HGT between eukaryotes and fungi primarily involve gene acquisition or transfer by fungi to or from other distantly related fungi or bacteria (Keeling and Palmer 2008; Keese 2008b) and HGT between plants and fungi is extremely rare (Richards et al. 2009). Examples of HGT between plants and invertebrates are also extremely rare, and most examples of HGT in insects involve acquisition of genes from their pathogens or endosymbionts (Keese 2008a; Zhu et al. 2011; Acuna et al. 2012).

Horizontal transfer from and expression in bacteria of the foreign DNA inserted into the nuclear genome of the GE plant is unlikely to occur. First, many genomes (or parts thereof) have been sequenced from bacteria that are closely associated with plants including *Agrobacterium sp.* and *Rhizobium sp.* (Wood et al. 2001; Kaneko et al. 2002). There is no evidence that these organisms contain genes derived from plants. HGT from plants to bacteria is a very low frequency event, primarily because functional and selective barriers to HGT increase with genetic distance (Keese 2008b). Second, in cases where review of sequence data implied that horizontal gene transfer occurred, these events are inferred to occur on an evolutionary time scale on the order of millions of years (Koonin et al. 2001; Brown 2003a; EFSA 2009a). Third, transgene DNA promoters and coding sequences are optimized for plant expression, not prokaryotic bacterial expression. Thus, even if horizontal gene transfer occurred, proteins corresponding to the transgenes are not likely to be produced. Fourth, both the FDA (US-FDA 1998) and the European Food Safety Authority (EFSA 2009b) have evaluated horizontal gene transfer from the use of antibiotic resistance marker genes and concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of humans or animals, or in the environment, is very rare or remote.

Potential for horizontal gene transfer to viruses

APHIS also considered whether horizontal transfer of DNA from the modified chestnut tree to plant viruses was likely to occur and would lead to the creation or selection of plant viruses that are more virulent or have a broader host range. This issue has been considered before by other science review panels and government regulatory bodies (Keese 2008b). HGT is not unusual among plant viruses; however this is generally limited to exchange between viruses present in the same host organism in mixed infections, and most commonly involves homologous recombination, relying on sequence similarity at the point of crossover (Keese 2008b). HGT from virus sequences engineered into plants has been demonstrated with infecting or challenge viruses, including both DNA viruses (e.g., geminiviruses that replicate in the nucleus) (Frischmuth and Stanley 1998) and RNA viruses (that typically replicate in the cytoplasm); however most have been under conditions that favor recombination to restore a defective virus (Thompson and Tepfer 2010). Populations of recombinants between virus transgenes expressed in transgenic plants infected with related viruses are similar to recombinants found in mixed infections of the same viruses in non-transgenic plants, indicating that there was no novel recombination mechanism in the transgenic plants and no increased risk is expected over what is expected from mixed infections (Keese 2008b; Turturo et al. 2008). Nonhomologous recombination in HGT among viruses or between virus transgenes and infecting viruses can occur, but frequently results in gene deletions that can result in nonviable viruses (Morroni et al. 2013). Depending on the particular virus and sequences involved, various hot-spots for recombination have been found in both coding and noncoding regions, and strategies implemented in design of transgenes to avoid recombination have been suggested. No recombinant or undesirable viruses with new properties have been detected for over at least 10 years in field tests or during commercial growth of deregulated virus resistant plum, squash, or papaya engineered with genes from viruses that have been deregulated in the U.S. (Fuchs and Gonsalves 2007).

The only virus sequence inserted in Darling 58 American chestnut is the 35S promoter for the cauliflower mosaic virus (CaMV) involved in regulating gene expression. CaMV is the type species of the genus Caulimovirus in the family Caulimoviridae – a group of double-stranded DNA viruses known as pararetroviruses, in which replication occurs in the cytoplasm via reverse transcription of an RNA intermediate. CaMV is a plant virus transmitted by sap inoculation, and in nature by aphids such as *Brevicoryne brassicae*, *Myzus persicae*, and at least 25 other species in a semi-persistent manner. Caulimoviruses generally have a narrow host range limited to plants of the family Brassicaceae, but some isolates are able to infect plants of the family Solanaceae experimentally (Hansen and Heslop-Harrison 2004). American chestnut is not known to be susceptible to CaMV or any Caulimoviruses.

Recombination in Caulimoviruses occurs predominantly, if not exclusively, in the cytoplasm by template switching between RNA transcripts during the replication process, although a low level of recombination involving viral DNA may occur in the nucleus (Froissart et al. 2005). Since the Caulimovirus promoter sequences are not transcribed in transgenic plants, there is little or no opportunity for them to recombine with any related

Caulimoviruses that may infect American chestnut. Based on the foregoing, horizontal transfer of DNA from Darling 58 American chestnut to plant viruses is unlikely to occur or is unlikely to lead to the creation or selection of plant viruses that are more virulent or have a broader host range.

Potential for horizontal gene transfer to parasitic plants

Evidence for HGT from plants to other plants is limited to two specific scenarios: (1) exchange of genes between a parasitic plant and its host; and (2) exchange of genes between cells of two plants living in close proximity, such as in a graft junction. In both cases, this type of HGT requires physical contacts between the two plants. Most cases of HGT in plants involve transfer of mitochondrial genomes, which are primarily maternally inherited in plants (Barr et al. 2005a, b), to other mitochondria genomes, and mostly involve parasitic plants and their hosts (Richardson and Palmer 2007). Recently, a comparative genomics analysis implicated HGT for the incorporation of a specific genetic sequence in the parasitic plant purple witchweed (*Striga hermonthica*) from its monocot host plant (Yoshida et al. 2010). According to this study, the incorporation of the specific genetic sequence (with an unknown function) occurred between sorghum and purple witchweed. However, this HGT occurred before speciation of purple witchweed and related cowpea witchweed (*S. gesnerioides*) from their common ancestor. More recent studies demonstrated that in a few parasitic species of the Rafflesiaceae family, out of several genetic sequences examined, about 2.1% of nuclear (Xi et al. 2012a) (Xi et al. 2012b)and 24 –41% of mitochondrial (Xi et al. 2013) gene transcripts appeared to be acquired from their obligate host species. However, all the above-mentioned instances of HGT between parasitic plants and their hosts were reported to be of ancient origins, on an evolutionary time scale spanning thousands to millions of years ago. Furthermore, in the GE crop, the DNA sequences were inserted into the nuclear genome, not the mitochondrial genome.

If the GE plant becomes infected by a parasitic plant or is naturally grafted to another plant, there is a very low probability that HGT could result in the other plant acquiring DNA from the GE plant. However, in both scenarios this newly introduced DNA would likely reside in somatic cells, and with little chance of reaching the germ cells, this introduced DNA could not persist in subsequent generations unless the recipient plant reproduced asexually from the affected cells.

Based on the above analysis, APHIS therefore concludes that HGT of the new genetic material inserted into Darling 58 American chestnut to other organisms with which it cannot interbreed is highly unlikely and is not expected to lead directly or indirectly to disease, damage, injury, or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

J. Conclusion

APHIS has reviewed the information submitted in the petition, supporting documents, public comments in response to the Federal Register notice concerning this petition, and

other relevant information to assess the plant pest risk of blight-tolerant Darling 58 American chestnut compared to the unmodified wild-type parent tree from which it was derived and other unmodified American chestnut controls. APHIS concludes that Darling 58 American chestnut is unlikely to pose a greater plant pest risk than its unmodified parent based on the following findings:

- No plant pest risk was identified from the transformation process or the insertion of new genetic material in Darling 58 American chestnut. The *Agrobacterium* transformation vector was disarmed and therefore lacked sequences from Tumor-inducing (Ti) plasmids normally responsible for the formation of galls in host plants of *A. tumefaciens*; transformed material was treated to kill the bacterium; a terminator sequence donated from *A. tumefaciens* and a promoter sequence donated from Cauliflower mosaic virus do not code for or express any gene products, and those inserted plant pest sequences do not cause disease or create infectious agents.
- No increase in plant pest risk was identified from expression of the inserted genetic material, the new Darling 58 OxO or NPTII proteins, or changes in metabolism or composition. The composition of Darling 58 American chestnut tissues were determined to be substantially equivalent to the wild-type parent tree from which Darling 58 was derived and other unmodified American chestnut controls. The mode of action and specificity of Darling 58 OxO or NPTII proteins raise no plant pest concerns.
- Disease and pest incidence and/or damage, other than reductions in chestnut blight from the target organism *Cryphonectria parasitica*, were not observed to be significantly different or atypical in Darling 58 American chestnut compared to the unmodified American chestnut controls in field trials conducted in growing regions representative of where Darling 58 American chestnut is expected to be grown**.** The oxalate oxidase blight tolerance and the neomycin phosphotransferase selectable marker traits did not significantly alter the response of Darling 58 to diseases or arthropod pests under natural levels of these stressors, and pest arthropods were not more abundant around Darling 58 plots compared to the unmodified controls. Observed agronomic traits also did not reveal any significant differences that would indirectly indicate that Darling 58 is more susceptible to pests or diseases**.** Therefore, no plant pest effects are expected on these or other forest or agricultural products and no impacts are expected to APHIS pest control programs.
- Exposure to and/or consumption of Darling 58 American chestnut is unlikely to have any adverse impacts on organisms beneficial to agriculture based on APHIS' analysis of studies on Darling 58 American chestnut food and feed safety, nutrient and anti-nutrient composition, levels of OxO and NPTII in tissues, environmental interactions with beneficial arthropods, and pollen characteristics.
- Darling 58 American chestnut is unlikely to become more of a weed or volunteer problem than wild-type American chestnut based on its observed agronomic characteristics, the low weediness potential of American chestnut, and current management practices available to control Darling 58 as a weed. Darling 58 American chestnut volunteers can be controlled with all currently available weed control methods, should a landowner wish to remove Darling 58 American chestnut from their property.

- Darling 58 American chestnut is not expected to increase the weed risk potential of other American chestnut, and other *Castanea* species with which it can interbreed. The genetic modification in Darling 58 American chestnut is not expected to increase its potential for gene flow, hybridization, and/or introgression to sexually compatible taxa, nor is it likely to increase their weediness potential in the event that such species were to be introduced. Introgression of the oxalate oxidase blight tolerance and neomycin phosphotransferase selectable marker traits into other American chestnut or related species will likely make them resistant to chestnut blight.
- Changes to silvicultural and orchard management practices (e.g., planting, pesticide applications, irrigation, nut harvesting, coppicing, timber harvesting, etc.) from adoption of Darling 58 American chestnut are only related to blight management practices and this is unlikely to increase pests or diseases or adversely impact their management, nor will they impact APHIS pest control programs.
- Horizontal gene transfer of the new genetic material inserted into Darling 58 American chestnut to other organisms is highly unlikely and is not expected to lead directly or indirectly to disease, damage, injury, or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

K. References

7 CFR 340. 2020. *Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There Is Reason to Believe Are Plant Pests*.

7 CFR 340. 2021. *Movement of Organisms Modified or Produced through Genetic Engineering*.

7 U.S.C. 136 *et seq.* 2019 Edition. *Federal Insecticide, Fungicide and Rodenticide Act.* . Retrieved from https://www.govinfo.gov/content/pkg/USCODE-2019-title7/pdf/USCODE-2019-title7-chap6-subchapII.pdf or https://www.agriculture.senate.gov/imo/media/doc/FIFRA.pdf

7 U.S.C. 7701 *et seq.* 2019. *Plant Protection Act*. Retrieved from https://www.govinfo.gov/content/pkg/USCODE-2019-title7/pdf/USCODE-2019-title7-chap104.pdf

21 U.S.C. 301 *et seq.* 2018 Edition. *Federal Food, Drug and Cosmetic Act* Retrieved from https://www.govinfo.gov/content/pkg/USCODE-2018-title21/pdf/USCODE-2018-title21-chap9.pdf

40 CFR part 152. 2019 Edition. *Pesticide Registration and Classification Procedures*. Retrieved from https://www.govinfo.gov/content/pkg/CFR-2019-title40-vol26/pdf/CFR-2019-title40-vol26-part152.pdf

40 CFR part 158. 2019 Edition. *Data Requirements for Pesticides*. Retrieved from https://www.govinfo.gov/content/pkg/CFR-2019-title40-vol26/pdf/CFR-2019-title40-vol26-part158.pdf

40 CFR part 172. 2019 Edition. *Experimental Use Permits*. Retrieved from https://www.govinfo.gov/content/pkg/CFR-2019-title40-vol26/pdf/CFR-2019-title40-vol26-part172.pdf
40 CFR part 174. 2019 Edition. *Procedures and Requirements for Plant Incorporated Protectants (PIPs)* Retrieved from https://www.govinfo.gov/content/pkg/CFR-2019-title40-vol26/pdf/CFR-2019-title40-vol26-part174.pdf
51 FR 23302. 1986. *Coordinated Framework for Regulation of Biotechnology*.
57 FR 22984. 1992. *Statement of Policy: Foods Derived from New Plant Varieties*.
Abrams MD. 2003. *Where Has All the White Oak Gone?* BioScience 53, pp. 927-939. Retrieved from https://doi.org/10.1641/0006-3568(2003)053[0927:WHATWO]2.0.CO;2 Last accessed 6/9/2022.
Acuna R, Padilla BE, Florez-Ramos CP, Rubio JD, Herrera JC, Benavides P, Lee S-J, Yeats TH, Egan AN, Doyle JJ, and Rose JKC. 2012. *Adaptive horizontal transfer of a bacterial gene to an invasive insect pest of coffee*. Proceedings of the National Academy of Sciences of the United States 109(11):4197-4202. Retrieved from http://www.pnas.org/content/early/2012/02/17/1121190109.full.pdf+html
Anagnostakis SL. 2012. *Chestnut breeding in the United States for disease and insect resistance*. Plant Disease 96, pp. 1392-1403. Retrieved from https://apsjournals.apsnet.org/doi/10.1094/PDIS-04-12-0350-FE
Baldry MGC. 1983. *The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid*. Journal of Applied Bacteriology 54, pp. 417-423. Retrieved from https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2672.1983.tb02637.x Last accessed 2018/06/08/21:11:39.
Barr CM, Neiman M, and Taylor DR. 2005a. *Inheritance and recombination of mitochondrial genomes in plants, fungi and animals*. New Phytologist 168, pp. 39-50. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1469-8137.2005.01492.x/abstract
Barr CM, Neiman M, and Taylor DR. 2005b. *Inheritance and recombination of mitochondrial genomes in plants, fungi and animals*. New Phytologist 168(1):39-50. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1469-8137.2005.01492.x/pdf
Binkley MA. 2008. *The phylogeography of North American Chestnuts and Chinquapins (Castanea, Mill., Fagaceae)*. The University of Tennessee at Chattanooga.
Brown JR. 2003a. *Ancient horizontal gene transfer*. Nature Reviews Genetics 4(2):121-132. Retrieved from http://www.nature.com/nrg/journal/v4/n2/full/nrg1000.html
Brown JR. 2003b. *Ancient horizontal gene transfer*. Nature reviews. Genetics 4, pp. 121-132. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12560809
CABI. 2022. *Invasive Species Compendium* Retrieved from https://www.cabi.org/ISC
Chakraborty J, Ghosal D, Dutta A, and Dutta TK. 2012. *An insight into the origin and functional evolution of bacterial aromatic ring-hydroxylating oxygenases*. Journal of Biomolecular Structure and Dynamics 30, pp. 419-436. Retrieved from https://doi.org/10.1080/07391102.2012.682208
Cook R and Forest HS. 1979. *The American chestnut II: Chestnuts in the Gennessee Valley region, 1978*. The Rochester Committee for Scientific Information Bull 226, pp. 1-9.

Dalgleish HJ, Nelson CD, Scrivani JA, and Jacobs DF. 2015. *Consequences of shifts in abundance and distribution of American chestnut for restoration of a foundation forest tree*. Forests 7, pp. 4. Retrieved from https://www.mdpi.com/1999-4907/7/1/4

Dane F. 2022. *Evolutionary History of American Castanea species*. The American Chestnut Foundation. Retrieved from https://acf.org/ct/news-and-updates/evolutionary-history-of-american-castanea-species/

Davis DE. 2005. *Historical Significance to American Chestnut to Appalachain Culture and Ecology*. Proceedings of Conference on Restoration of American Chestnut to Forest Lands, pp. 1-8.

Davis MB. 1981. Quaternary History and the Stability of Forest Communities. In: *Forest Succession* (New York, NY: Springer New York), pp. 132-153. Retrieved from http://link.springer.com/10.1007/978-1-4612-5950-3_10 Last accessed 2019/03/07/14:41:40.

Davis MB. 1983. *Quaternary History of Deciduous Forests of Eastern North America and Europe*. Annals of the Missouri Botanical Garden 70, pp. 550-563. Retrieved from http://www.jstor.org/stable/2992086 Last accessed 2019/08/11/19:19:32.

Dröge M, Puhler A, and Selbitschka W. 1998. *Horizontal gene transfer as a biosafety issue: A natural phenomenon of public concern*. Journal of Biotechnology 64, pp. 75–90. Retrieved from http://www.sciencedirect.com/science/article/pii/S0168165698001059

Dumitru R, Jiang WZ, Weeks DP, and Wilson MA. 2009. *Crystal Structure of Dicamba Monooxygenase: A Rieske Nonheme Oxygenase that Catalyzes Oxidative Demethylation*. Journal of Molecular Biology 392, pp. 498-510. Retrieved from https://www.sciencedirect.com/science/article/pii/S0022283609008614

EFSA. 2009a. *Consolidated presentation of the joint Scientific Opinion of the GMO and BIOHAZ Panels on the "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants" and the Scientific Opinion of the GMO Panel on "Consequences of the Opinion on the Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants on Previous EFSA Assessments of Individual GM Plants*. The European Food Safety Authority Journal 7(1108):1-107. Retrieved from http://www.efsa.europa.eu/en/efsajournal/pub/1108

EFSA. 2009b. *Statement of EFSA on the consolidated presentation of opinions on the use of antibiotic resistance genes as marker genes in genetically modified plants*. EFSA Journal 1108. Retrieved from http://www.efsa.europa.eu/en/efsajournal/pub/1108.htm

Ellstrand NC, Prentice HC, and Hancock JF. 1999. *Gene Flow and Introgression from Domesticated Plants into their Wild Relatives*. Annual Review of Ecology and Systematics 30, pp. 539-563. Retrieved from https://www.annualreviews.org/doi/abs/10.1146/annurev.ecolsys.30.1.539

Emily WBR. 1987. *Pre-Blight Distribution of Castanea dentata (Marsh.) Borkh*. Bulletin of the Torrey Botanical Club 114, pp. 183-190. Retrieved from http://www.jstor.org/stable/2996129 Last accessed 2022/05/26/.

State University of New York College of Environmental Science and Forestry. 2019. *PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR BLIGHT-TOLERANT DARLING 58 AMERICAN CHESTNUT (Castanea dentata)*

Submitted by W.A. Powell, Ph.D. . State University of New York College of Environmental Science and Forestry. Syracuse, New York.
Faison EK and Foster DR. 2014. *Did American Chestnut Really Dominate the Eastern Forest?* Arnoldia 17, pp. 18-32.
Frischmuth T and Stanley J. 1998. *Recombination between viral DNA and the transgenic coat protein gene of African cassava mosaic geminivirus*. Journal of General Virology 79, pp. 1265-1271. Retrieved from https://jgv.microbiologyresearch.org/content/journal/jgv/10.1099/0022-1317-79-5-1265#tab2
Froissart R, Roze D, Uzest M, Galibert L, Blanc S, and Michalakis Y. 2005. *Recombination Every Day: Abundant Recombination in a Virus during a Single Multi-Cellular Host Infection*. PLOS Biology 3, pp. e89. Retrieved from https://doi.org/10.1371/journal.pbio.0030089
Fuchs M and Gonsalves D. 2007. *Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies*. Annual Review of Phytopathology 45, pp. 173-202. Retrieved from http://www.annualreviews.org/doi/full/10.1146/annurev.phyto.45.062806.094434?url_ver=Z39.88-2003
Fulbright D, Stadt S, Medina-Mora C, Mandujano M, Donis-González I, and Serdar U. 2012. *Kernel breakdown appears when hybrid Castanea cultivars are pollinized by Castanea mollissima*.
GBIF. 2022. *GBIF Occurrence Download https://doi.org/10.15468/dl.kcn87d*.
Gepts P and Papa R. 2003. *Possible effects of (trans)gene flow from crops on the genetic diversity from landraces and wild relatives*. Environmental Biosafety Research 2, pp. 89-103. Retrieved from https://www.ebr-journal.org/articles/ebr/pdf/2003/02/E3206.pdf
Grant V. 1981. *Plant Speciation, 2nd Edition*. New York: Columbia University Press, 563 pp.
Grant V. 1994. *Modes and origins of mechanical and ethological isolation in angiosperms*. Proceedings of the National Academy of Sciences 91(1):3-10. Retrieved from http://www.pnas.org/content/91/1/3.full.pdf+html
Guilley H, Dudley RK, Jonard G, Balàzs E, and Richards KE. 1982. *Transcription of cauliflower mosaic virus DNA: detection of promoter sequences, and characterization of transcripts*. Cell 30, pp. 763-773. Retrieved from http://www.sciencedirect.com/science/article/pii/0092867482902811 Last accessed 2018/03/22/20:14:54.
Hansen C and Heslop-Harrison JS. 2004. Sequences and Phylogenies of Plant Pararetroviruses, Viruses, and Transposable Elements. In: *Advances in Botanical Research* (Academic Press), pp. 165-193. Retrieved from https://www.sciencedirect.com/science/article/pii/S0065229604410040
Hegde SG, Nason JD, Clegg JM, and Ellstrand NC. 2006. *The evolution of California's wild radish has resulted in the extinction of its progenitors*. Evolution 60(6):1187-1197. Retrieved from http://dx.doi.org/10.1554/05-634.1
Hellens R, Mullineaux P, and Klee H. 2000. *Technical focus: A guide to Agrobacterium binary Ti vectors*. Trends in Plant Science 5(10):446-451. Retrieved from http://plant-tc.cfans.umn.edu/listserv/2002/log0205/pdf00000.pdf

Hoekema A, Hirsch PR, Hooykaas PJJ, and Schilperoort RA. 1983. *A binary plant vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid*. Nature 303:179-180. Retrieved from http://www.nature.com/nature/journal/v303/n5913/abs/303179a0.html

Jacobs DF, Dalgleish HJ, and Nelson CD. 2013. *A conceptual framework for restoration of threatened plants: the effective model of American chestnut (Castanea dentata) reintroduction*. New Phytologist 197, pp. 378-393.

Jacobs DF, Dalgleish HJ, and Nelson CD. 2016. *Synthesis of American chestnut (Castanea dentata) biological, ecological, and genetic attributes with application to forest restoration*. Forest Health Initiative (http://foresthealthinitiative. org/resources/chestnutdossier. pdf). Accessed online 7.

Jaynes RA and DePalma NK. 1984. *Natural Infection of Nuts of Castanea dentata by Endothia parasitica*. Phytopathology 74, pp. 296-299. Retrieved from https://www.apsnet.org/publications/phytopathology/backissues/Documents/1984Articles/Phyto74n03_296.PDF

Kaneko T, Nakamura Y, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, Watanabe A, Idesawa K, Iriguchi M, Kawashima K, Kohara M, Matsumoto M, Shimpo S, Tsuruoka H, Wada T, Yamada M, and Tabata S. 2002. *Complete genomic sequence of nitrogen-fixing symbiotic bacterium Bradyrhizobium japonicum USDA110*. DNA Research 9(6):189-197. Retrieved from http://dnaresearch.oxfordjournals.org/content/9/6/189.long

Keeling PJ and Palmer JD. 2008. *Horizontal gene transfer in eukaryotic evolution*. Nature reviews. Genetics 9, pp. 605-618. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18591983

Keese P. 2008a. *Risks from GMOs due to horizontal gene transfer*. Environmental Biosafety Research 7(3):123-149. Retrieved from http://journals.cambridge.org/action/displayAbstract?fromPage=online&aid=8208895&fileId=S1635792208000146

Keese P. 2008b. *Risks from GMOs due to horizontal gene transfer*. Environmental Biosafety Research 7, pp. 123-149. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/18801324

Khoury CK, Greene S, Wiersema J, Maxted N, Jarvis A, and Struik PC. 2013. *An Inventory of Crop Wild Relatives of the United States*. Crop Science 53, pp. 1496-1508.

Koonin EV, Makarova KS, and Aravind L. 2001. *Horizontal gene transfer in prokaryotes: Quantification and classification*. Annual Review of Microbiology 55:709-742. Retrieved from http://www.annualreviews.org/doi/full/10.1146/annurev.micro.55.1.709?url_ver=Z39.88-2003

Lang P, Dane F, and Kubisiak TL. 2006. *Phylogeny of Castanea (Fagaceae) based on chloroplast trnT-L-F sequence data*. Tree Genetics & Genomes 2, pp. 132-139. Retrieved from https://doi.org/10.1007/s11295-006-0036-2

Little ELJR. 1977. Castanea dentata (Marsh.) Borkh.. American chestnut. In: *Atlas of United States Trees* (Washington, DC: United States Department of Agriculture Forest Service).

Long S. 2012. *Midwest Nut Producers Council Journal*. 2012 1, pp. 3.

McEwan RW, Keiffer CH, and McCarthy BC. 2006. *Dendroecology of American chestnut in a disjunct stand of oak chestnut forest*. Canadian Journal of Forest Research 36, pp. 1-11.

Morroni M, Jacquemond M, and Tepfer M. 2013. *Deep sequencing of recombinant virus populations in transgenic and nontransgenic plants infected with Cucumber mosaic virus*. Molecular Plant-Microbe Interactions 26, pp. 801-811. Retrieved from http://apsjournals.apsnet.org/doi/abs/10.1094/MPMI-02-13-0057-R?url_ver=Z39.88-2003

Newhouse AE, Oakes AD, Pilkey HC, Roden HE, Horton TR, and Powell WA. 2018. *Transgenic American Chestnuts Do Not Inhibit Germination of Native Seeds or Colonization of Mycorrhizal Fungi*. Frontiers in Plant Science 9, pp. 1-9.

Nowacki GJ and Abrams MD. 2008. *The Demise of Fire and "Mesophication" of Forests in the Eastern United States*. BioScience 58, pp. 123-138. Retrieved from http://academic.oup.com/bioscience/article/58/2/123/259756/The-Demise-of-Fire-and-Mesophication-of-Forests-in Last accessed 2019/03/19/19:18:26.

Paillet FL and Rutter PA. 1989. *Replacement of native oak and hickory tree species by the introduced American chestnut (Castanea dentata) in southwestern Wisconsin*. Canadian Journal of Botany 67, pp. 3457-3469. Retrieved from http://www.nrcresearchpress.com/doi/abs/10.1139/b89-423 Last accessed 2014/05/28/20:37:17.

Peng R-H, Xiong A-S, Xue Y, Fu X-Y, Gao F, Zhao W, Tian Y-S, and Yao Q-H. 2010a. A Profile of Ring-hydroxylating Oxygenases that Degrade Aromatic Pollutants. In: *Reviews of Environmental Contamination and Toxicology Volume 206* (New York, NY: Springer New York), pp. 65-94. Retrieved from https://doi.org/10.1007/978-1-4419-6260-7_4

Peng R-H, Xiong A-S, Xue Y, Fu X-Y, Gao F, Zhao W, Tian Y-S, and Yao Q-H. 2010b. *A profile of ring-hydroxylating oxygenases that degrade aromatic pollutants*. Reviews of Environmental Contamination and Toxicology 206:65-94. Retrieved from http://link.springer.com/chapter/10.1007/978-1-4419-6260-7_4

Perkins MT, Robinson AC, Cipollini ML, and Craddock JH. 2019. *Identifying Host Resistance to Phytophthora cinnamomi in Hybrid Progeny of Castanea dentata and Castanea mollissima*. HortScience horts 54, pp. 221-225. Retrieved from https://journals.ashs.org/hortsci/view/journals/hortsci/54/2/article-p221.xml

Preston CD, Pearman DA, and Dines TD. 2002. *New Atlas of the British & Irish Flora*. Oxford University Press, 928 pp.

Ramputh AI, Arnason JT, Cass L, and Simmonds JA. 2002. *Reduced herbivory of the European corn borer (Ostrinia nubilalis) on corn transformed with germin, a wheat oxalate oxidase gene*. Plant Science 162, pp. 431-440. Retrieved from http://www.sciencedirect.com/science/article/pii/S0168945201005842 Last accessed 2013/07/09/18:58:14.

Reichard SH and Hamilton CW. 1997. *Predicting Invasions of Woody Plants Introduced into North America*. Conservation Biology 11, pp. 11.

Richards TA, Soanes DM, Foster PG, Leonard G, Thornton CR, and Talbot NJ. 2009. *Phylogenomic analysis demonstrates a pattern of rare and ancient horizontal gene transfer between plants and fungi*. Plant Cell 21, pp. 1897-1911. Retrieved from http://www.plantcell.org/content/21/7/1897.long

Richardson AO and Palmer JD. 2007. *Horizontal gene transfer in plants*. Journal of Experimental Botany 58, pp. 1-9. Retrieved from http://jxb.oxfordjournals.org/content/58/1/1.long

Rieseberg LH. 1997. *Hybrid origins of plant species*. Annual Review of Ecology and Systematics 28:359-389. Retrieved from http://www.annualreviews.org/doi/abs/10.1146/annurev.ecolsys.28.1.359

Rieseberg LH and Wendel JF. 1993. Introgression and its consequences in plants. In: *Hybrid Zones and the Evolutionary Process* (Oxford University Press), pp. 70-109.

Rogstad SH and Pelikan S. 2014. *Restoring the American Chestnut: Optimizing Founder Spacing to Promote Population Growth and Genetic Diversity Retention*. Restoration Ecology 22, pp. 668-675. Retrieved from https://onlinelibrary.wiley.com/doi/abs/10.1111/rec.12124 Last accessed 2018/09/14/19:02:10.

Rutter P. 1990. *Chestnut pollinator's guide*. Badgersett Research Corporation Bulletin 1.

Sandercock A, Westbrook J, Zhang Q, Johnson H, Saielli T, Scrivani J, Fitzsimmons S, Collins K, Schmutz J, Grimwood J, and Holliday J. 2022. *Whole-genome resequencing reveals the population structure, genomic diversity, and demographic history of American chestnut (Castanea dentata)*. bioRxiv, pp. 2022.2002.2011.480151. Retrieved from http://biorxiv.org/content/early/2022/02/13/2022.02.11.480151.abstract

Shaw J, Craddock JH, and Binkley MA. 2012. *Phylogeny and Phylogeography of North American Castanea Mill. (Fagaceae) Using cpDNA Suggests Gene Sharing in the Southern Appalachians (Castanea Mill., Fagaceae)*. Castanea 77, pp. 186-211. Retrieved from http://www.castaneajournal.org/doi/abs/10.2179/11-033 Last accessed 2016/06/01/13:28:18.

Sisco P, Hebard F, Craddock J, Shaw J, and Neel T. 2012. *Cytoplasmic male sterility in interspecific hybrids between American and Asian Castanea species is correlated with the American D chloroplast haplotype*.

Snow AA. 2002. *Transgenic crops—why gene flow matters*. Nature Biotechnology 20, pp. 542-542. Retrieved from https://doi.org/10.1038/nbt0602-542

Soltis DE, Soltis PS, and Rieseberg LH. 1993. *Molecular data and the dynamic nature of polyploidy*. Critical Reviews in Plant Sciences 12(3):243-273. Retrieved from http://www.tandfonline.com/doi/abs/10.1080/07352689309701903#.UhekuRtwq-w

Soucy SM, Huang J, and Gogarten JP. 2015. *Horizontal gene transfer: building the web of life*. Nature Reviews Genetics 16, pp. 472-482. Retrieved from https://doi.org/10.1038/nrg3962

Stace CA. 1987. Hybridization and the plant species. In: *Differentiation Patterns in Higher Plants* (New York: Academic Press), pp. 115-127.

Tasei J-N and Aupinel P. 2008. *Nutritive value of 15 single pollens and pollen mixes tested on larvae produced by bumblebee workers (Bombus terrestris, Hymenoptera: Apidae)*. Apidologie 39, pp. 397-409.

Thompson DV and Tepfer M. 2010. *Assessement of the Benefits and Risksfor Engineered Virus Resistance*. Advances in Virus Research 76, pp. 33-55.

Turturo C, Friscina A, Gaubert S, Jacquemond M, Thompson JR, and Tepfer M. 2008. *Evaluation of potential risks associated with recombination in transgenic plants expressing viral sequences*. The Journal of General Virology 89, pp. 327-335. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/18089757

US-FDA. 1998. *Guidance for Industry: Use of Antibiotic Resistance Marker Genes in Transgenic Plants*.

US-FDA. 2006. *Guidance for Industry: Recommendations for the Early Food Safety Evaluation of New Non-Pesticidal Proteins Produced by New Plant Varieties Intended for Food Use*.

US-FDA. 2022. *Biotechnology Consultations on Food from GE Plant Varieties.* Retrieved from https://www.fda.gov/food/consultation-programs-food-new-plant-varieties/recently-published-biotechnology-consultations

USDA-APHIS. 2022. *Petitions for Determination of Nonregulated Status* Retrieved from https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions/petitions/petition-status

USDA-NRCS. 2022. *USDA Plants Database search for Castanea sp.* . Retrieved from https://plants.usda.gov/home/basicSearchResults?resultId=5f6b6c62-02eb-495f-9366-a695f0d5af83

Wang GG, Knapp BO, Clark SL, and Mudder BT. 2013. *The Silvics of Castanea dentata (Marsh.) Borkh., American chestnut, Fagaceae (Beech Family)*. Southern Research Station. Retrieved from https://www.fs.usda.gov/treesearch/pubs/43054 Last accessed 2018/01/25/18:16:13.

Welch AJ, Stipanovic AJ, Maynard CA, and Powell WA. 2007. *The effects of oxalic acid on transgenic Castanea dentata callus tissue expressing oxalate oxidase*. Plant Science 172, pp. 488-496. Retrieved from https://www.sciencedirect.com/science/article/pii/S0168945206003001

Wood DW, Setubal JC, Kaul R, Monks DE, Kitajima JP, Okura VK, Zhou Y, Chen L, Wood GE, Almeida NF, Jr., Woo L, Chen Y, Paulsen IT, Eisen JA, Karp PD, Bovee D, Sr., Chapman P, Clendenning J, Deatherage G, Gillet W, Grant C, Kutyavin T, Levy R, Li MJ, McClelland E, Palmieri A, Raymond C, Rouse G, Saenphimmachak C, Wu Z, Romero P, Gordon D, Zhang S, Yoo H, Tao Y, Biddle P, Jung M, Krespan W, Perry M, Gordon-Kamm B, Liao L, Kim S, Hendrick C, Zhao ZY, Dolan M, Chumley F, Tingey SV, Tomb JF, Gordon MP, Olson MV, and Nester EW. 2001. *The genome of the natural genetic engineer Agrobacterium tumefaciens C58*. Science 294:2317-23. Retrieved from http://www.sciencemag.org/content/294/5550/2317.long

WSSA. 2022. *Composite List of Weeds* Retrieved from https://wssa.net/wssa/weed/composite-list-of-weeds/

Xi S, Bradley RB, Wurdack KJ, Wong KM, Sugumaran M, Bomblies K, Rest JS, and Davis CC. 2012a. *Horizontal transfer of expressed genes in a parasitic flowering plant*. BMC genomics. Retrieved from http://www.biomedcentral.com/1471-2164/13/227

Xi Z, Wang Y, Bradley RK, Sugumaran M, Marx CJ, Rest JS, and Davis CC. 2013. *Massive mitochondrial gene transfer in a parasitic flowering plant clade*. PLoS Genetics 9. Retrieved from

http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003265

Xi Z, Bradley RK, Wurdack KJ, Wong K, Sugumaran M, Bomblies K, Rest JS, and Davis CC. 2012b. *Horizontal transfer of expressed genes in a parasitic flowering plant*. BMC Genomics 13:227. Retrieved from http://www.biomedcentral.com/1471-2164/13/227

Yoshida S, Maruyama S, Nozaki H, and Shirasu K. 2010. *Horizontal Gene Transfer by the Parasitic Plant Striga hermonthica*. Science 328, pp. 1128.

Zhu B, Lou M-M, Xie G-L, Zhang G-Q, Zhou X-P, Li B, and Jin G-L. 2011. *Horizontal gene transfer in silkworm, Bombyx mori*. BMC Genomics 12:248. Retrieved from http://www.biomedcentral.com/1471-2164/12/248

Zon R. 1904. *Chestnut in southern Maryland*. Washington, DC: US Department of Agriculture, Bureau of Forestry.