

State University of New York College of Environmental Sciences and Forestry Petition (19-309- 01p) for Determination of Nonregulated Status for Blight-Tolerant Darling 54 American Chestnut

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Draft Plant Pest Risk Assessment

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TABLE OF CONTENTS

Contents

TABLE OF CONTENTS	PAGE
A. Introduction.....	3
B. Development of Blight-Tolerant Darling 54 American Chestnut.....	5
C. Description of Inserted Genetic Material, Its Inheritance and Expression, Gene Products, and Changes to Plant Metabolism.....	7
D. Potential Plant Pest and Disease Impacts	9
E. Potential Impacts on Nontarget Organisms Beneficial to Agriculture.....	11
F. Potential for Enhanced Weediness of Darling 54 American Chestnut	12
G. Potential Impacts on the Weediness of Any Other Plants with which Darling 54 American Chestnut Can Interbreed	13
H. Potential Changes to Silviculture or Agriculture Practices.....	16
I. Potential Impacts from Transfer of Genetic Information to Organisms with which Darling 54 American Chestnut Cannot Interbreed.....	20
J. Conclusion	23
K. References	25

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-DAR54-4” (hereafter referred to as Darling 54 American chestnut) developed using genetic engineering (hereafter referred to as modified chestnut) for enhanced blight tolerance. The petition presents evidence supporting the argument that Darling 54 American chestnut is unlikely to pose a plant pest risk and, therefore, should no longer be regulated under APHIS’ 7 Code of Federal Regulations (CFR) part 340. This petition was assigned the number 19-309-01p and is hereafter referenced as ESF 2024 (ESF 2024). Under the authority of the plant pest provisions of the Plant Protection Act (7 U.S.C. 7701 *et seq.* 2024), the regulations in 7 CFR part 340, “Introduction of Organisms and products altered or produced through genetic engineering which are plant pests or which there is a reason to believe are plant pests,” 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 plant pest risk. This plant pest risk assessment (PPRA) was conducted to determine if the Darling 54 American chestnut is unlikely to pose a plant pest risk.

Based on supplemental information received from the petitioner on May 24, 2022, APHIS posted the availability of a Draft Environmental Impact Statement and Draft Plant Pest Risk Assessment in 2022. Subsequently APHIS received a revised petition (event name correction to Darling 54 for the earlier event name Darling 58) for determination of nonregulated status for blight-tolerant Darling 54 American Chestnut and supplemental data was received on August 9, 2024, and September 30, 2024, respectively.

Darling 54 American chestnut was produced by *Agrobacterium tumefaciens*-mediated transformation of somatic embryos from the isogenic line of Darling 54 known as Ellis using plasmid p35S-OxO (ESF 2024). Portions of the inserted genetic material were derived from plant pest organisms listed in 7 CFR § 340.2 (i.e., a promoter sequence from cauliflower mosaic caulimovirus (CaMV) and a terminator sequence from *A. tumefaciens*) (Section 7.1, ESF 2024). Therefore, Darling 54 American chestnut is considered regulated under APHIS regulations at 7 CFR part 340 (7 CFR 340 2020). ESF has conducted field releases of Darling 54 American chestnut under APHIS authorizations since 2014 (ESF 2024), in part, to gather information to support that Darling 54 American Chestnut is unlikely to pose a greater plant pest risk than the nonmodified 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 54 American chestnut and its progeny and their use in the absence of confinement relative to the nonmodified recipient line and/or other appropriate comparators. APHIS utilizes data and information submitted by the applicant, in addition to current literature, to determine if Darling 54 American chestnut is unlikely to pose a greater plant pest risk than the nonmodified organism from which it was

derived. APHIS regulations in 7 CFR § 340.6(c) specify the information needed for consideration in a petition for nonregulated status. APHIS will assess the information submitted by the applicant about Darling 54 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.* 2024), 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.* 2024). 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 2024). Other applicable EPA regulations include Pesticide Registration and Classification Procedures (40 CFR part 152 2024), Procedures and Requirements for Plant Incorporated Protectants (PIPs) (40 CFR part 174 2024), and Experimental Use Permits (40 CFR part 172 2024). ESF informed APHIS that EPA has deemed the expressed OxO protein in their Darling 54 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 54 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 54 American chestnut and is awaiting FDA's response.

B. Development of Blight-Tolerant Darling 54 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 2024). 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 harvested for timber. Specimen trees were 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 extinct in modern forests (ESF 2024).

To rescue and ultimately restore American chestnut in its native environment, the petitioner has developed Darling 54 American chestnut trees with enhanced tolerance to chestnut blight. Darling 54 American chestnut was developed from a single immature zygotic embryo extracted from a wild-type American chestnut seed. The embryo was first cultured to produce a 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. 83, ESF 2024). 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. 83, ESF 2024). 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. Putative modified 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 2024).

The transformed American chestnut plant (Darling 54) expresses the *oxo* gene encoding oxalate oxidase (OxO) that neutralizes oxalic acid produced by the chestnut blight fungus. (ESF 2024) Additionally, Darling 54 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 2024).

The isogenic recipient genome of Darling 54 is a wild-type *C. dentata* tissue culture line known as Ellis that was established from a single immature zygotic embryo extracted from the wild seed of an American chestnut tree (*C. dentata* (Marsh.) Borkh.) known as Pond #1 (ESF 2024).

Control materials used as comparators in the Darling 54 safety assessments include the isogenic recipient line Ellis and nonmodified 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 54 leaf litter, insect herbivory on Darling 54 leaves, bumble bee foraging on Darling 54 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 2024).

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 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 copies of inserted genetic material in the modified plant.

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 54 American chestnut compared to the isogenic and other nonmodified controls. The assessment encompasses a consideration of the expressed oxalate oxidase (OxO) from *Triticum aestivum*, the expressed neomycin 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 derived from Darling 54 American chestnut compared to those in the isogenic or other nonmodified 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 Darling54 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 2024):

- **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 neomycin phosphotransferase (NPTII) from *Escherichia coli*.
- **Tnos: 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 54 American chestnut. 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 2024):

- The T-DNA inserted into the Darling 54 American chestnut genome is present at a single locus of 4.53 kilobases in size, on chromosome 4, within a DNA sequence homologous to the *Sal1* phosphatase-like gene found in other plants.
- Darling 54 has a small deletion (approximately 1,051 base pairs) and an inversion (approximately 5 base pairs) in a specific region of chromosome 4 (Figure 7.2a). The deletion is not shown in the figure; however, it is described in the text.
- The T-DNA sequence in Darling 54 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 54 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 tissues. Oxalate oxidase is widely present in many food grains wild plants and microbes, and it is well understood with a history of over 100 years' studies (Li et al. 2022; ESF 2024). Wheat oxalate oxidase enzyme has been well characterized and can effectively detoxify oxalate (Li et al. 2022). Since wheat is consumed daily by humans and livestock, there are no known safety concerns associated with the consumption of this enzyme (ESF 2024).

The oxalate oxidase gene in Darling 54 was found to be expressed in stem and leaf tissues, with expression levels decreasing in that order. Moreover, enzyme activity of oxalate oxidase (OxO) was measured in the stem, leaf, root, and nut tissues of Darling 54, revealing a similar decreasing trend across these tissues. (Figure 7.4.2a, ESF 2024).

The petitioner carried out the compositional analysis, including nutritional contents, of Darling 54 chestnuts and nonmodified American chestnuts to assess whether levels of

key nutrients and compositions in Darling 54 American chestnut were equivalent to levels in the nonmodified control. The analysis showed that Darling 54 and nonmodified nuts have nearly nutritional profiles based on FDA guidelines for food labels. While there are some differences in nutritional measurements of American chestnut from various source trees and among similar trees over different years, the values of modified chestnuts are within the range found in nonmodified chestnuts. This supports the conclusion that Darling 54 American chestnut is nutritionally and compositionally similar to its related nonmodified controls.

The petitioner states that the T-DNA sequence, which contains the p35S-OxO gene, was inserted within a SalI phosphatase-like gene. This gene belongs to a family of highly similar genes found in the American chestnut genome. A search of the 'Ellis1' genome on Phytozome, using the Arabidopsis SalI phosphatase sequence (AT5G63980) as a query, revealed over a dozen such genes. APHIS also analyzed the chestnut DNA sequences flanking the T-DNA insertion using the Phytozome database and confirmed that the insertion occurred within a gene similar to the SalI-phosphatase gene in Arabidopsis. The Ellis 1 chestnut genome contains 10 genes closely related to this SalI-phosphatase-like gene. These potentially paralogous genes may provide functional redundancy, mitigating any harmful effects from the disruption of one or more related genes. Also, intercrosses between transgenic trees have generated healthy, morphologically normal individuals homozygous for the Darling 54 insertion. This demonstrates that the insertion site itself does not cause significant detrimental effects. Furthermore, the genetic alteration introduced by this T-DNA insertion is minimal compared to the genetic rearrangements often observed in traditional breeding (Section 2.1.2, ESF 2024).

The genetic modification in Darling 54 also resulted in a small deletion of about 1,051 base pairs and a tiny inversion of just 5 base pairs in a specific region of its DNA (Figure 7.2a). These types of changes—deletions, inversions, and rearrangements—happen naturally all the time, both in wild populations and during traditional breeding. In fact, research suggests that average rearrangements in transgenic plants are likely *smaller* than the changes due to natural rearrangements that occur during traditional breeding methods (Dane et al. 2015; Anderson et al. 2016; Schouten et al. 2017; Bashir et al. 2018).

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 American chestnut plant metabolisms except the intended blight tolerance trait compared to nonmodified 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 54 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 54 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 54 American chestnut. The binary plasmid vector p35S-OxO proved to be disarmed; the T-DNA inserted into Darling 54 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 54 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 54 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 54 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 54 American chestnut compared with the nonmodified controls.

Data from these tests showed that the most substantial difference in Darling 54 American chestnuts compared to the nonmodified controls is its oxalic acid tolerance that allows the trees to coexist with the blight fungus. However, Darling 54 American chestnuts are not associated with increased susceptibility or tolerance to specific abiotic stressors, arthropods, or diseases compared to controls (Section 9 and 10, ESF 2024). The results support the conclusion that the blight tolerance trait in Darling 54 American chestnut is not expected to alter the response of Darling 54 to diseases or arthropod pests under natural levels of these stressors, nor cause pest arthropods to be more abundant around Darling 54 plots, compared to nonmodified controls.

In summary, the introduced genes did not significantly alter the observed insect pest infestation and disease occurrence or resulting damage on Darling 54 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 54 is or could be more susceptible to pests and diseases compared to the control lines. Thus, Darling 54 is unlikely to be more susceptible to plant pathogens and insect pests than nonmodified American chestnut and existing commercial hybrids, and it is unlikely to differ from nonmodified 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 54 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 54 American chestnut compared to the nonmodified 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 54, as well as additional literature.

As indicated earlier in this plant pest risk assessment, the mechanism of the blight defense in Darling 54 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 54

showed nutrient composition levels in nuts were comparable to the nonmodified control, and that Darling 54 OxO expression level is low, so there is unlikely to be nontarget effects resulting from changes in composition or from consumption of Darling 54 OxO. Also, the study on environmental interactions found that there were no changes in beneficial arthropod abundance in field plots of Darling 54.

Therefore, based on the above analysis of ESF's studies on Darling 54 nut composition, levels of OxO in tissues, and environmental interactions with beneficial arthropods, APHIS concludes exposure to and/or consumption of Darling 54 are unlikely to have any adverse impacts on organisms beneficial to agriculture.

F. Potential for Enhanced Weediness of Darling 54 American Chestnut

APHIS assessed whether Darling 54 is likely to become weedier than the nonmodified 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 54 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, competitiveness, 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 a competitive growth rate, it exhibits intermediate shade tolerance, and the 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 2024).

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 2024). However, American chestnut takes at least several years to produce seeds, and a good number of seeds would be produced only after the 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 2024). 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 2024). 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 2024). These data indicate that Darling 54 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 54 and wild-type American chestnut, it is implied that Darling 54 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 54 American chestnut populations establish in non-native habitats.

G. Potential Impacts on the Weediness of Any Other Plants with which Darling 54 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 and Soltis 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 54 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 (Russell 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 occurrence 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 2.1a 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, ESF 2024). 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. (Dalglish et al. 2016). The pollen flow from Darling 54 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. (2013) 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 54 to the wild relatives would be possible because *Castanea* species can hybridize freely (Lang et al. 2006). Also, there are reports of natural occurrence of interspecific hybrids such as *C. dentata* x *C. mollissima* and *Castanea* 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 54 American chestnut and other *Castanea* species.

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

Darling 54 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 detoxifies 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 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 54 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 relatives are not listed as weeds or invasive species by any U.S. 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 54 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 nonmodified recipient or other varieties of chestnut that are commonly growing in forests and in silviculture settings. The genetic modification in Darling 54 American chestnut is unlikely to confer novel weedy properties to chestnut or its wild relatives. Therefore, APHIS has determined that Darling 54 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 54 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 54 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. ESF plans to cross Darling 54 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 54 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 54 leaf litter, insect herbivory on Darling 54 leaves, bumble bee foraging on Darling 54 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 2024).

Phenotypic observations (Section 8, ESF 2024) including growth and photosynthesis and environmental interaction experiments on Darling 54 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 54 is not significantly different from nonmodified chestnut controls (Appendix I, ESF 2024).

Phenotypic characteristics

ESF evaluated phenotypic characteristics as compared to nonmodified and traditionally bred controls in field, laboratory, or greenhouse experiments. Additionally, data from legacy modified event, Darling 4, provides 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 nonmodified control trees.

Furthermore, the data support the conclusion that the Darling 54 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 the oxalic acid/blight tolerance trait that allows chestnut trees to coexist with the blight fungus.

Environmental Interactions

Mycorrhizal colonization of Darling 54 roots

There were no significant differences in colonization by ectomycorrhizal fungi in roots compared to nonmodified controls. These results suggest that presence or expression of OxO in Darling 54 does not pose novel pest risks to native soil fungi that are ecologically

important for American chestnuts and other trees (ESF 2024). The introduction of Darling 54 back into eastern forests is unlikely to have negative impacts on soil quality.

Responses of plants found in chestnut habitats: germination of seeds in Darling 54 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. nonmodified 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 nonmodified 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 nonmodified controls. Darling 54 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 2024).

Responses to other pests and environmental stresses

Apart from chestnut blight tolerance, no differences between modified and nonmodified trees have been observed in response to plant-disease, plant-arthropod, or plant-environment interactions. Modified and nonmodified 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 54 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 54 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 54 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 54 American chestnut and nonmodified American chestnut

Persistence of OxO activity in Darling 54 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 54 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 2024). 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 54 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 54 trees will continue to produce nonmodified, 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 54 is a hemizygous event with only one copy of the inserted *oxo* gene present in the genome. Thus, a cross with such hemizygous modified tree as a parent will always result in the offspring without transgene. Even when two Darling 54 trees are crossed, about 25% of their offspring would be nonmodified and blight-susceptible (Section 6.4, ESF 2024). 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 54 American chestnut is similar to nonmodified controls in its agronomic, phenotypic, environmental response and compositional characteristics and has levels of pests and diseases or their damage levels comparable to nonmodified American chestnuts. Furthermore, Darling 54 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 54 trees will include both plants with transgene and plants without transgene, the original wild-type American chestnut will be conserved in the future (ESF 2024). In conclusion, APHIS could not

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

I. Potential Impacts from Transfer of Genetic Information to Organisms with which Darling 54 American Chestnut Cannot Interbreed

APHIS examined the potential for the new genetic material inserted into Darling 54 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 modified organisms to another organism without reproduction or human intervention have been reviewed (Keese 2008). 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 2008; 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 2003; Keeling and Palmer 2008; Keese 2008).

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

Darling 54 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. 2010). 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. 2010).

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 2008). 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 2008) 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 2008; 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 2008). 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 2003; EFSA 2009). 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 2009) 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 2008). 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 2008). 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 modified plants infected with related viruses are similar to recombinants found in mixed infections of the same viruses in nonmodified plants, indicating that there was no novel recombination mechanism in the modified plants and no increased risk is expected over what is expected from mixed infections (Keese 2008; 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 54 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

modified 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 54 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. 2005), 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. 2012) 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 54 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 54 American chestnut compared to the nonmodified wild-type parent tree from which it was derived and other nonmodified American chestnut controls. APHIS concludes that Darling 54 American chestnut is unlikely to pose a greater plant pest risk than its nonmodified 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 54 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 54 OxO or NPTII proteins, or changes in metabolism or composition. The composition of Darling 54 American chestnut tissues was determined to be substantially equivalent to the wild-type parent tree from which Darling 54 was derived and other nonmodified American chestnut controls. The mode of action and specificity of Darling 54 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 54 American chestnut compared to the nonmodified American chestnut controls in field trials conducted in growing regions representative of where Darling 54 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 54 to diseases or arthropod pests under natural levels of these stressors, and pest arthropods were not more abundant around Darling 54 plots compared to the nonmodified controls. Observed agronomic traits also did not reveal any significant differences that would indirectly indicate that Darling 54 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 54 American chestnut is unlikely to have any adverse impacts on organisms beneficial to agriculture based on APHIS' analysis of studies on Darling 54 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 54 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 54 as a weed. Darling 54 American chestnut volunteers can be controlled with all currently available weed control methods, should a landowner wish to remove Darling 54 American chestnut from their property.

- Darling 54 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 54 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 54 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 54 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.

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