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

(Executive Summary and Table of Contents)

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Executive Summary

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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