January 26, 2023

Animal and Plant Health Inspection Service
Biotechnology Regulatory Services
4700 River Road
Riverdale, MD 20737-1238

RE: Docket No. APHIS-2020-0030 State University of New York College of Environmental Science and Forestry; Availability of a Draft Environmental Impact Statement and Draft Plant Pest Risk Assessment for Determination of Nonregulated Status for Blight-Tolerant Darling 58 American Chestnut (*Castanea dentata*) Developed Using Genetic Engineering

Overview

On behalf of The American Phytopathological Society (APS), we respectfully submit these comments in response to the draft environmental impact statement and draft plant pest risk assessment which discussed impacts that may result from possible de-regulation and potential environmental release of the blight-tolerant Darling 58 American chestnut. Under the Coordinated Regulatory Framework for Agricultural Biotechnology, there are three agencies responsible for oversight: USDA's Animal and Plant Health Inspection Service (USDA-APHIS), the U.S. Environmental Protection Agency (EPA)[1], and the Department of Health and Human Services' Food and Drug Administration (FDA). Upon review of the documents, our membership of dedicated scientists offers constructive feedback to APHIS for consideration. Furthermore, we hope that our remarks will help clarify and streamline regulations in service of a science- and risk-based, predictable, efficient, and transparent system to support the safe use of products of biotechnology.[2]

APS is the premier professional society dedicated to high quality, innovative science for management of plant pathogens, pests and diseases of crop and forest trees. We are a distinctive community of scientists from academia, industry, government, and private practice, with a strong commitment to ensure the global advancement of phytopathology. Plant pathology is an interdisciplinary science that integrates knowledge of botany, microbiology, crop science, soil science, ecology, genetics, biochemistry, molecular biology, and plant physiology to understand pathogenesis and host resistance or tolerance to pathogens. Our members work closely with agencies such as APHIS on cutting-edge plant biology research initiatives. Through these efforts, we are working to promote the judicious use of

[1] The U.S. EPA has determined that the gene (and its associative genetic material), oxalate oxidase is consistent with the legal definition of a pesticide. Furthermore, it is legally defined as a Plant-Incorporated Protectant (PIP) because the source (wheat) of the active ingredient is not sexually compatible with the recipient plant , American chestnut. The petitioner announced this determination at the APS meetings in Pittsburgh, PA, August 6-9, 2022.

[2] Executive Order 14081:
https://usbiotechnologyregulation.mrp.usda.gov/biotechnologygov/home/modernizing/modernizing_biotechnology_framework

sound science to shape public policy as it relates to the study and management of crop and forest diseases.

Introduction

Blight-tolerant Darling 58 represents a new breed of American chestnut (*Castanea dentata*) trees, which have been engineered with a trait to enhance blight-tolerance. This trait is generated by a single gene from wheat(oxalate oxidase) which can be passed on to subsequent generations through classical Mendelian inheritance. By transferring specific genetic material from another plant (wheat) that is not sexually compatible with the recipient plant (American Chestnut), the petitioner has created blight-tolerant plants that produce proteins or other chemicals that the American Chestnut could not previously produce. Therefore, the plant-incorporated protectants are chemicals produced by plants whose DNA has been modified, as well as the DNA that produces the chemicals. The plant's modified DNA now expresses protective properties by producing a plant protein that will protect the American Chestnut. Furthermore, oxalate oxidase is normally found in many plant species and degrades oxalic acid to produce carbon dioxide and hydrogen peroxide, also found ubiquitously in plants. Oxalate oxidase does not kill *C. parasitica*; hence the inserted gene is more of a (plant-incorporated) protectant, and not a pesticide.

The purpose of these genetically engineered trees is to help rescue and restore extant populations of American chestnut, following introgression of the blight tolerance trait into a viable and diverse restoration population from their offspring. Because offspring of Darling 58 trees are expected to include both transgenic and non-transgenic individuals, the original wild-type American chestnut is expected to be conserved into the future.

The terms resistance and tolerance as used in the petition (Sections 4,5 and 8) are generally consistent with scholarly, peer-reviewed sources in the discipline of Plant Pathology. Resistance is understood as restriction of pathogen activity by the host, whereas tolerance is when the host endures pathogen activity while generally performing well. Both phenomena may be present in the same pathosystem. This distinction is important because, to the extent tolerance is operative, there is less selection pressure on the pathogen to evolve in response so as to overcome the presence and activity of the pathogen. In contrast, host factors which restrict pathogen activity (=resistance) create selection pressure and commonly result in the emergence of virulent strains which can overcome the resistance trait.[3]

A brief literature review in regards to breeding for blight-resistant American chestnut, and current biological control approaches using hypovirulence follow. Individual comments relevant to assessing potential consequences to the environment as a result of potential deregulation of the transgenic blight-tolerant American chestnut (Event Name: Darling 58 and offspring) are provided. A brief description of the scientific approach and methodology for creating *Agrobacterium tumefaciens (At)*-mediated gene transfer is also included.

[3] Resistance and tolerance terminology section provided by Paul Vincelli, Extension Professor, Univ. of Kentucky

Environmental Assessment

Forest ecosystems are subject to numerous abiotic and biotic stressors: windstorms, drought, wildfire as well as populations of insect pests and pathogenic microorganisms. Whereas some tree species may survive and eventually adapt to these stressors through ecological successional processes, exotic pathogens such as the chestnut blight fungus, *Cryphonectria parasitica* = *Endothia parasitic*a) threatens the continued existence of a species, because co-evolution processes with the introduced pathogen have not yet occurred. Clumps of small American chestnut trees exist in many of the extant populations within the former native range of American chestnut, and sightings of large surviving American Chestnut trees [22-114 cm diameter @ breast height] have been reported (Griffin, et al. 1983). Canopied large surviving American Chestnuts are rare.

Since *C. parasitica* survives on other hosts, and lethal susceptibility in the American chestnut is nearly universal, rescue and eventual restoration of the American chestnut may require incorporating extra specific alleles or genes through interspecific hybridization, or through genetic transformations of the host (biotechnology) or biocontrol of the pathogen (e.g., through cytoplasmic hypovirulence or through hypovirus disease of *Cryphonectria parasitica*).

Breeding for blight resistance has been a primary focus for restoration of the American chestnut for nearly a century (1930s to present) in the U.S. Since 1983, the American Chestnut Foundation (TACF) has been actively involved in establishing and managing Chinese (*C.mollisima*) x American (*C. dentata*) chestnut developed through a back-cross breeding program, as proposed by Charles Burnham in 1981 (Hebard, 2006). The only other active breeding program (Steiner et al., 2017) is based upon evaluation of native blight resistance developed from intercrossings of large, surviving American chestnut trees combined with the use of hypovirulence (*Cryphonectria Hypovirus*) *in situ* (Griffin, et al. 2006). Robbins and Griffin (1999) reported spread of dsRNA-containing hypovirulent strains (=cytoplasmic hypovirulence) 12 to 13 years post-inoculation into natural cankers on American Chestnut. Photographs of surviving chestnuts may be found on the website for the American Chestnut Cooperators Foundation (https://accf-online.org).

Development of blight -resistant all-American chestnut began in the early 1980s; some of the progeny from those controlled intercrossings have survived for 30 or years or more (American Chestnut Cooperators 'Foundation-ACCF, 2010; Griffin, unpublished data). The authors concluded that this survival was associated with resistance (perhaps additive), hypovirulence and favorable sites for American chestnut growth. In 2006, Griffin, *et al* reported that a high level of blight control was obtained on mesic, managed (control of competing hardwoods) sites, established with blight-resistant American chestnuts that were inoculated with a hypovirulent strain mixture.

Use of Hypovirulent *Cryphonectria parasitica* to control/manage chestnut blight[4]

Hypovirulence is the process of the chestnut blight hypovirus (H-Cp) infecting *C. parasitica* thus causing the fungus to be less debilitating to the trees and the trees are able to heal themselves (Grente and Berthel-Sauret, 1978). Hypovirulence does not usually spread in the US from tree to tree due to diversity within the fungal strains (Anagnostakis and Waggoner, 1981; Robbin and Griffin, 2002; Stauder et al, 2019); however research Hogan and Griffin , (2002) indicated that the spread of H-Cp and tree host resistance factors were associated with blight control. They also showed that the H-Cp strain used in

[4] Ms Daniella Mikolajewski, PhD candidate at West Va University contributed to this section.

their studies was present in 48 vegetative compatibility types of *C. parasitica*, which indicate that white-pigmented H-Cp had a high fitness for spread with a random or nearly random spatial pattern on the grafted American Chestnut trees. Cultural studies and nucleotide sequence analysis of two hypovirus regions (both >800 bp) indicated that blight control was associated with the spread of Italian C. Hypovirus 1 (CHV1) (Griffin et al., 2006). *In situ* investigations were conducted in areas that had abundant and virulent inoculum of *C. parasitica.*

Long-term application of hypoviruses in an infected stand of re-located (introduced outside its natural range) American chestnut in Wisconsin (Double, *et al*., 2018) provided evidence of biological control in locations where there are limited numbers of vegetative compatibility types of *C. parasitica*. Isolation of hypovirus-infected strains increased from 55% in 1994 to 86% in 2014 from cankers treated 17 years earlier . Treatments were administered in two consecutive five-year treatments: (a) from 1992-1997, and (b) from 2000-2014. Over a 23 year period, tree survivorship was 51% for trees with treated cankers, compared to 31% for trees with untreated cankers.

West Virginia University (WVU) has been working on super-donor strains that will increase hypovirulence spread providing more control of the fungus (Zhang and Nuss, 2016; Stauder et al, 2019). WVU has found some *C. parasitica* strains that cannot have hypovirulence spread to them and are investigating a possible new *C. parasitica* gene preventing spread of hypovirulence. Current research also involves hyphal anastomosis of H-Cp strains to determine the success of transmission of the hypovirus to create super-donor H-Cp.

Horizontal Gene Flow[5]

Concerns have been expressed as to whether the use of *Agrobacterium tumefaciens (At)* for plant transformation may introduce the risk of uncontrolled spread of *At* genomic fragments in natural ecosystems. A relevant paper may help to address these concerns (Kyndt, Quispe et al. 2015). In this paper, the authors presented evidence of T-DNA regions in the genomes of 291 cultivated varieties of sweet potato, as a result of natural domestication (Hallerman and Grabau 2016). Some of these fragments were shown to be expressed. These fragments were not found in close wild relatives of this crop, even though they go back in evolutionary time, seemingly providing sufficient opportunity for natural dissemination by either plant-to-plant hybridization or by transformation and integration into genomes of wild relatives.

Description of *At*-plant transformation and insertion of oxalate-oxidase gene[6]

One of the most commonly used methods of plant transformation involves the use of a "disarmed" *A. tumefaciens* (*At*) strain, that is, a strain of *At* with engineered deletions that render it incapable of causing crown galls. Such disarmed strains remain capable of attaching to plant cells made accessible by the wound and the *At* strain injects plasmid DNAs carrying foreign genes of interest through a pilus into the plant cell to which it is attached. Once in the plant cell, this DNA is guided to the plant nucleus, where some of it is integrated at random sites into a plant chromosome. The injected foreign DNA encodes a selectable antibiotic marker as well as a screenable fluorescence marker, plus a gene of interest, in this case, oxalate oxidase. The wounded tissue is then placed on a medium containing the

[5] This section drafted by Paul Vincelli, Extension Professor, University of Kentucky, and Jeff Jones, Professor, University of Florida.

[6] This section was drafted by Dean Gabriel, Professor of Plant Pathology & Program in Plant Molecular and Cell Biology, University of Florida.

antibiotic or herbicide, and only the transformed plant cell(s) with the resistance marker can survive and grow. The disarmed *At* strain used to deliver the DNA is killed by including an antibiotic specifically designed to kill *At,* but not affecting plant cells.

Since most plant cells, even in differentiated tissues, can be totipotent under the right conditions, those cells can be induced to regenerate complete plants from the single transformed plant cell with the integrated foreign DNA. (The capacity to regenerate plants from a single cell in this manner varies widely between plant species and even varieties). Each regenerated plant from a single transformed cell constitutes a single transformation "event", and nearly all of these cells regenerate plants that carry random genetic variation, often caused by chromosomal rearrangements. Therefore, although one might expect clones of the original parent plant, in practice each event produces a slightly different plant with different random mutations. This is one of the most important reasons for outcrossing to a wild-type parent, while maintaining selection for the engineered trait of interest..

According to the petition, a wild type American chestnut (*Castanea dentata*) tissue culture line was transformed with *At* and resulted in event "ESF-DAR58-3" (=Darling 58). This singular event has a single characterized insertion on *C. dentata* Chromosome 7 and lacks any *At* sequences or plasmid backbone sequences. It expresses the commonly used kanamycin resistance enzyme neomycin phosphotransferase (NPTII). It also carries a commonly used promoter region from CaMV and a nopaline synthase terminator and left and right T-DNA border regions from *At*. It expresses oxalate oxidase from wheat. It should be noted that oxalate oxidase is not a pesticide*, sensu stricto* rather protects against oxalate that is produced by pathogens [i.e., allows the host to 'tolerate 'the fungal pathogen].

Outcrossing (or hybridization) of the oxalate oxidase gene to various selected WT American chestnut trees will likely need to be carried out by conventional means to create T1 progeny from the Darling 58 T0 founding event. The idea is to incorporate much needed resistance from the Darling 58 event to wild type American chestnut trees adapted to different locations (Sandercock et al. 2022) and to eliminate the inevitable random somaclonal mutations in Darling 58 caused by the process of regeneration during transformation. Mendelian inheritance predicts that the T1 plants carrying the transgene will contain only 50% Darling 58 alleles. If another outcross is performed, then this will result in T2 progeny with only 25% Darling 58 alleles. Additional outcrosses, each time with selection of the kanamycin resistance marker will result in half again the number of Darling 58 alleles.

Historically, disarmed *At* was first used to transform poplars in 1987 (Fillatti et al., 1987), representing the first forest species to be transformed. One of the first practical applications using transformation in a tree species was moving the *Bacillus thuringiensis* (*Bt*- endotoxin into *Populus* spp (McCown et al., 1991); later in similar work, Robinson et al (1994) reported nearly complete protection from the larvae of gypsy moth and of forest tent caterpillar for some events.

<u>Outcrossing and Subsequent Gene Flow to Wild Relatives</u>

Cross-pollination (gene flow) of wild relatives may disrupt a local ecosystem by changing the landscape of local plants, by competing with related species, and/or by changing the habitat[7].

[7] https://www.epa.gov/regulation-biotechnology-under-tsca-and-fifra/epas-regulation-biotechnology-use-pest-management#testing

Outcrossing (via open or uncontrolled pollination) and subsequent gene flow from transgenic pollen to wild relatives is expected to be high since plant breeders had discovered all chestnut species to be cross-fertile. Seven taxonomic *Castanea* species are currently recognized: *dentata*, *sativa*, *mollisima*, *crenate*, *segunii*, *pumila* var. *pumila*, *pumila* var. *ozarkensis* and *henryi* (respectively American, European, Chinese, Japanese, Chinese Dwarf Chinquapin*, A*merican Chinquapin or Bush Chestnut, Ozark Chinquapin and Henry Chinquapin). One possible wild hybrid between *C. dentata* and *C. pumila* (*Castanea X neglecta* Dode) was also included in the list of wild *Castanea* species (Anagnostakis and Hillman, 1992).

The petitioners aim to increase the genetic diversity and adaptive capacity remaining in extant and autothonous *C. dentata* populations. Uncontrolled pollination of transgenic trees with non-transgenic wild-type (WT) *Castanea* spp. is of serious concern to organic chestnut growers and to orchardists or foresters in the National Park Service who are working to breed blight-resistant All American chestnut trees or wild chinquapin trees for restoration and/or private use. Restoration plantings and efforts reported by the ACCF (2010) include:

- National, State Forest & National Park Lands (Since 1980 to present): ACCF locations in Jefferson National Forest, Lesesne State Forest, VA; Mammoth Cave National Park, KY

- Private and State lands: (1976 - present): ACCF research having mostly controlled pollination progeny of F1, F2, and/or F3 generations in Giles, Montgomery and Nelson Counties, VA; Raleigh Co, WV; Humphreys Co, TN

- ACCF Cooperators (citizens, federal and state foresters, university researchers, et al): Locations are in natural range of American chestnut (in almost all eastern states of US), having mostly open-pollination production, although some have controlled pollination progeny from ACCF. Somatic seedlings have been produced and planted in 2010 in cooperation with Scott Merkle (University of Georgia). Sandra Anagnostakis (Connecticut Agricultural Experiment Station) and Scott Schlarbaum (University of Tennessee) have field tests of ACCF progeny.

Vegetative hybridization techniques (e.g., grafting) was used extensively in previous breeding programs at the Connecticut Agricultural Experiment Station. The ACCF also prefers grafting germ plasm from progeny of large surviving chestnut trees before releasing to their cooperators (Griffin, 2006; ACCF website), because gene flow and exchange are better controlled with minimal risk of gene flow from other wild chestnut species. Chestnut pollen is readily wind-borne and in orchard or forest plantation settings could also be insect-vectored.

<u>Potential impacts to non-target organisms and to the forest ecosystem</u>.

Oxalate oxidase is normally found in many plant species, especially in cereal crops. It degrades oxalic acid to produce two common compounds, carbon dioxide and hydrogen peroxide, also found ubiquitously in plants. Oxalate oxidase is a major component of germinating seeds and is also referred to as germin. It is a naturally occurring protein in many foods, such as breads, cereals, and malt products, to name a few, and is safely consumed daily by millions around the world. As pointed out in the petition, it is non-toxic and non-allergenic. The authors also state that “it is not a pesticide”. In the scientific context of disease resistance or tolerance mechanisms, it probably acts as a (plant-incorporated) protectant, as it may be involved in mitigating a specific pathogenicity mechanism of the necrotrophic pathogen. Oxalate oxidase does not kill *C. parasitica*.

Oxalate may play a dual role in *C.parastica* pathogenesis: as a synergist with polygalacturonase, advancing tissue maceration; it was also found to be toxic toward protoplasts, aiding in acidifying the canker, especially along the advancing edge of the mycelium (McCarroll and Thor, 1978).

The composition of the nuts from the transgenic trees was examined for nutrition and tannin content and showed no differences compared to non-transgenic counterparts. Furthermore, genetic engineering is not inherently riskier than other methods of plant improvement, and perhaps even less so due to the precision of the genetic engineering techniques. Traditional breeding approaches include crossing (hybridization) with other species and mutational breeding (intentional production of mutations by use of radiation or chemical mutagens). These methods introduce many more, and potentially deleterious, changes in the plant genome than the introduction of a well-characterized OXO transgene. These traditional methods are not subject to regulatory oversight. Genome sequences for American chestnut (Sandercock, et al 2022) provides an additional route to examine transgenic plants for any potential changes compared to the non-transgenic plant.

Our membership has direct experience in the use of oxalate oxidase as a transgene and in the examination of transgenic plant lines. One of our members 'laboratories – Dr. Beth Grabau's – used OXO to confer tolerance to the fungal pathogen *Sclerotinia minor* in multiple cultivars of peanut (Blight Blocker peanuts). They examined many of the same characteristics in peanuts that were studied for in the American chestnut and found no differences from non-transgenic peanuts in those studies (6 publications, several of which were referenced in the petition). Laboratory and field trials showed successful reduction of disease, not elimination of the pathogen. OXO transgene is now stably inherited through T12 generation. Kernel composition and quality and plant physiology were assessed for Blight Blocker cultivars and were nearly identical for transgenic versus non-transgenic lines. A low outcrossing potential of Blight Blocker peanuts was quantified. Blight Blocker peanuts were also examined for comparative susceptibility to other (non-target) pathogens and showed that the addition of the OXO transgene did not have any negative impacts on susceptibility to other pathogens, which is important for both crop plants and other species.[8]

One of the concerns with genetically modified organisms is that transformation and related processes can result in unexpected and unintended phenotypic changes, potentially altering ecological interactions. Summaries of laboratory, greenhouse and field evaluations studies conducted to examine potential environmental impacts on mycorrhizal colonization, native plant species, aquatic and terrestrial insects, and tadpoles. No significant differences were observed for OXO-expressing American chestnut compared to non-transgenic lines. Transgenic chestnut does not have a significant effect on allelopathic mycorrhizal communities, seed germination of other trees, shrubs and grasses grown nearby, or herbivore communities that interact with trees. Moreover, leaf litter from the transgenic American chestnut did not have any negative effects on wood frog larvae development, a common sympatric species with the American chestnut. Likewise, the growth and physiology of transgenic chestnut are similar to those of the non-transgenic trees. These results suggest that the transgenic American chestnut does not pose greater ecological risks than conventionally bred trees. [9]

[8] This section was drafted by Beth Grabau, Professor Emeritus , Department of Plant Pathology, Physiology and Weed Science, Va Tech,

[9].Olga Kozhar, Postdoctoral researcher in the Department of Agricultural Biology, Colorado State University contributed portions of this paragraph.

It is noted that full reports of these studies are cited in the petition and are available as science publications, reports, posters, or theses.

Concluding Remarks

We have provided several comments above which address a few issues associated with the regulatory review for the new breed of American Chestnut, Blight-tolerant Darling 58. The science as proposed in the petition is solid, well-documented, and appears consistent with present understanding of the pathogenesis of *Cryphonectria parasitica* and its roles in resistance/tolerance mechanisms in American chestnut survival. The previous and ongoing research with hypovirulent *C. parasitica* offers additional opportunities for study of biological management of chestnut blight, particularly in autothonous, or relocated American chestnut populations having a wide range of genetic diversity.

It should be noted that the National Park Service (NPS) has specific policy guidance regarding pest management. It relies on integrated pest management (IPM) which allows use of a chemical, biological, or bio-engineered pesticide in a management strategy following a determination by a designated IPM specialist that such use is necessary, and that all other available options are either not acceptable or not feasible (Dennis, J. 2006).

It is beyond the scope of this commentary to discuss the NPS policy guidance for restoring American chestnut in NPS units. Summarily, any restoration project of this magnitude and scope would need to be based on a clear understanding by all participants of the scientific basis for, and the methodological requirements of each of the potential approaches to blight control/management.

Furthermore, volunteers are expected to play large roles in many aspects of a reforestation program; the two organizations mentioned in this commentary depend heavily upon ‘citizen scientists ’to accomplish many of the physical, horticultural or silvicultural aspects of this extensive and ambitious program., expected to span a few decades or more.

APS appreciates the opportunity to provide feedback to APHIS on this critical issue. We look forward to further opportunities to work with APHIS on these impactful regulatory decisions.

Thank you,

Ron Walcott

Ron Walcott
APS President

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