



## CENTER FOR FOOD SAFETY

19 Oct 2020

### **Petition for Determination of Nonregulated Status for Blight-tolerant Darling 58 American Chestnut (*Castanea dentata*)**

Event name: Darling 58 and offspring  
Tentative OECD Unique Identifier: ESF-DAR58-3  
Docket: APHIS-2020-0030

Comments and supporting materials submitted electronically:  
<http://www.regulation.gov/#!docketDetail;D=APHIS-2020-0030>

Center for Food Safety and International Center for Technology Assessment appreciate the opportunity to comment on this Petition.

Blight-tolerant American chestnut (*Castanea dentata*) Darling 58 cultivar is genetically engineered with an oxalate oxidase (OxO) gene from wheat intended to allow offspring that receive it to survive infections from the ascomycete fungus *Cryphonectria parasitica* well enough that the trees can become dominant overstory species in forests again. This is a project to rapidly domesticate a wild species through genetic engineering and accelerated breeding, and then to put it back into ecosystems to form self-perpetuating populations (Westbrook et al 2020 at 94) – an intentional evolutionary intervention that has never been attempted before with any species. The large spatial scale throughout eastern North America (Petition at 25, 27) and long timeline of more than a century to gauge success (e.g. Petition at 39-40) requires that this unprecedented experiment be carefully evaluated before deregulation by the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA).

The researchers at State University of New York College of Environmental Science and Forestry (ESF), under the direction of William Powell, who developed Darling 58 have prepared and submitted a Petition for Determination of Nonregulated Status (Petition), which APHIS has made public and available for comment (Federal Register/Vol. 85, No. 161/Wednesday, August 19, 2020/Notices: 51008-09 (FR Notice)).

APHIS will use the Petition and information submitted during this comment period to determine whether Darling 58 should continue to be regulated under 7 CFR part 340 rules. (“Data were gathered on multiple parameters and used by the applicant to evaluate agronomic characteristics and product performance. These and other data are used by APHIS to determine if the new variety poses a plant pest risk.” FR Notice at 51008)

**WASHINGTON D.C. OFFICE**  
660 Pennsylvania Avenue, SE Suite 402  
Washington, D.C. 20003  
T: 202-547-9359 F: 202-547-9429

**CALIFORNIA OFFICE**  
303 Sacramento Street, 2nd Floor  
San Francisco, CA 94111  
T: 415-826-2770 F: 415-826-0507

**PACIFIC NORTHWEST OFFICE**  
2009 NE Alberta Street, Suite 207  
Portland, OR 97211  
T: 971-271-7372

In addition, APHIS will assess impacts “in accordance with the National Environmental Policy Act (NEPA), to provide the Agency with a review and analysis of any potential environmental impacts associated with the petition request” in order to prepare an environmental assessment (EA) or an environmental impact statement (EIS)(FR Notice at 51009). Public comments are solicited to inform this process. (“We are interested in receiving comments regarding potential environmental and interrelated economic issues and impacts that APHIS may determine should be considered in our evaluation of the petition.” FR Notice at 51008)

### **Overview: not enough data to assess risks of deregulating Darling 58**

There are not enough observations and data in the Petition for APHIS to be able to assess plant pest risks and environmental, health and other impacts of Darling 58. Petitioners claim that, “[a]s described in this petition, Darling 58 has been studied in detail and no plant pest or environmental risks have been observed.” (Petition at 18; more at 101). However, Darling 58 was first regenerated from somatic embryo cultures in 2013 (Petition at 74) and has only been planted from seeds in field trials since 2017 (Petition at 82, 231). The Petition was submitted in January of 2020. This means that the oldest seed-grown Darling 58 trees available for study had been in the field for at most 3 growing seasons.

It is well documented that the phenotype of each transformation event must be observed and analyzed throughout the plant’s life stages in relevant environmental conditions. Even when the same DNA is inserted there can be significant differences in patterns of transgene expression that affect function (Latham et al 2006, Wilson et al 2006). Also, steps of the genetic engineering process introduce random genetic and epigenetic changes (Neelakandan and Wong 2012, Miguel and Marum 2011). It is standard practice for these differences between events to be evaluated by biotechnologists before using them to meet intended goals.

The stated goal of the petitioners is ecological restoration of American chestnut by returning it to its pre-blight dominant canopy position in forests. American chestnut trees before blight could live several hundred years, reaching large sizes (“...Zon (1904) reports that chestnut trees live to an age of 400 to 600 years, though trees over 100 years grow hollow in the center. American chestnut trees were commonly recorded at heights of 70 to 100 feet, with diameters of 3 to 5 feet or more ...”, Petition at 31).

It is thus premature to be petitioning for Darling 58’s nonregulated status after 3 seasons of field trials. Experiments and observations based on such young trees are unsuitable for making plant pest risk assessments or for determining environmental impacts of unregulated release. American chestnut trees do not become fully susceptible to blight until they are about 5 years old (“These tests were short-term evaluations of very young trees (<4 years old) in a relatively uniform environment. Juvenile resistance may be affecting results...”, Clark et al 2014 at 508; “Natural blight infections have only rarely been observed on Darling 58 trees due to their age and size to date (the largest Darling 58 trees alive at the time of writing are about 3 years old)...”, Petition at 77) Female flowers do not develop until trees are about 5 years under ideal conditions and usually longer, and it is likely to be 20 years before a reliable nut crop is produced (Petition at 33). The architecture of Darling 58 as it matures must be described to assess competitive ability

and potential to join the canopy as intended. Chemical and physical characteristics of roots, leaves, bark, branches, and the tree trunk change as the trees mature, which can affect interactions with other organisms (Lindenmayer and Laurance 2016, 2017).

In addition, most studies in the Petition related to environmental assessments, such as toxicity of leaves to tadpoles and aquatic insects, were done with earlier OxO gene transformation events, not with Darling 58, using the concept of “bridging” (“Bridging refers to the use of data from other transgenic events intended to support conclusions on a primary event.” Petition at 15; use of different events is summarized in Table 1.3a, although some information is apparently in error on the table, e.g. non-transgenic pollen, not Darling 58 pollen, was used in bumble bee studies, see Petition at 138 and discussion below).

Bridging is not sound science when, as here, the earlier events have different regulatory elements and transgene expression patterns, transgene copy numbers, and genetic backgrounds (Petition at 96, Section 10) and have not themselves been assessed and approved for deregulation by APHIS.

Potential restoration of the American chestnut to forests of eastern North America is described as a project that will take centuries (Petition at 39-40). Keeping Darling 58 regulated until there are sufficient data for meaningful assessments is necessary at this early stage in the project. The Petition should be withdrawn or denied on that basis.

If APHIS goes ahead with PPRA and NEPA assessments in spite of insufficient information to do so, impacts that APHIS must analyze and consider in deciding whether and how to approve or deny the petition include, but are not limited to, the following examples.

### **Issues and impacts for APHIS to consider in its Plant Pest Risk Assessment and National Environmental Policy Act analysis of the proposed deregulation of Darling 58**

#### *Impacts of Darling 58 based on level of tolerance to *Cryphonectria parasitica**

Chestnut blight is caused by the exotic fungal pathogen *C. parasitica* that no previous role in healthy forests of North America and since its introduction around 1900 has devastated American chestnut throughout its range (Petition at 46-48). Field trials since 2006 of genetically engineered American chestnut OxO transformation events, as described in the Petition, show that in some circumstances the OxO gene from wheat allows American chestnut trees to grow and reproduce in the presence of blight while continuing to host cankers with fungal hyphae and spores that are infective. This is described as “tolerance” rather than “resistance” to blight (Petition at 64).

One of the oldest OxO-engineered trees is growing under permit at the New York Botanical Garden, Bronx, NY, and illustrates the phenomenon of tolerance to blight (Petition at 159, Figure 10.5.1b, reproduced below.) Transplanted to that site in 2012, this Darling 4 tree was perhaps 8-10 years old in the photo. Although still alive without dying back, it is evident that large cankers cover the trunk. Whether this tree will mature in such a way that it can become part of the canopy in eastern forests of North America is not known.



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

There are no comparable photos of Darling 58 trees at any age in the Petition for visual comparison. Unlike Darling 58, Darling 4 is engineered with an OxO gene construct meant to express only in vascular tissues rather than in all tissues of the tree, and levels of expression are much lower than in Darling 58 (e.g. Petition at 96, leaf expression compared). Darling 58 has

higher tolerance to blight in early tests (Petition at 101-103), correlated with higher OxO expression, but at this point there are no data from direct observations to confirm that Darling 58 will have substantially better tolerance to blight than Darling 4 as it matures in natural settings (Petition at 147).

APHIS must consider impacts of different scenarios, including the possibility that Darling 58 trees will not grow tall enough to reach the canopy or will not live long enough to fulfill the historical ecological role of American chestnut trees. Impacts of earlier senescence could be wide-ranging (e.g. accelerating climate change by prematurely decreasing carbon sinks, Brienen et al 2020).

Chestnut blight cankers have their own ecologies, with different kinds of fungi and strains of blight coming and going in the same canker over time (Kolp et al 2020), and many species of insects are attracted to cankers as they change (Russin et al 2014). How will the ecology of cankers on Darling 58 compare with other infected chestnut trees, and what are the implications of any differences? Tolerance to blight based on OxO may interact with other virulence factors, as well (Lovat and Donnelly 2019 at 10, 21). Observations are needed as Darling 58 trees experience natural infections over a period of years.

The impact of blight-tolerant Darling 58 trees at various stages of growth on levels of *C. parasitica* spore levels in the forest and persistence of blight (tolerance described: Petition at 147, 65, 76-77) that can potentially impact future chestnut restoration efforts must be evaluated (e.g. recovery projects as in Clark et al 2014, Woodcock et al 2018, Boland et al 2012, Dalglish et al 2016).

#### Responses of pests and pathogens other than *Cryphonectria parasitica* to Darling 58

A suite of serious pests and pathogens, many of them introduced during global trade, plague American chestnut today that threaten success of restoration (Wang et al 2013, Clark et al 2014). Observations on how Darling 58 responds to other pests and pathogens have been made on young plants. Comparisons were made to non-transgenic isogenic lines growing close by in growth rooms, greenhouses, and field trials. Also, inferences were made based on earlier events, such as Darling 4 with much lower levels of OxO that also might be confined to vascular tissue. There have been no targeted pest surveys (“... all these observations are informal and anecdotal...”, Petition at 183). Studies where Darling 58 trees are challenged with various pests and pathogens in well-designed experiments are required to draw meaningful conclusions.

When evaluating plant pest risks in agricultural settings, increases in plant pests and pathogens are generally assumed to be entirely detrimental. In non-agricultural environments, though, many co-evolved plant pests and pathogens are integral parts of ecosystems and are required for healthy functioning of the whole. For example, endemic soil pathogens that infect and kill seedlings close to mother trees are important arbiters of tree biodiversity in forests (Bagchi et al 2014, Bever et al 2015, Jia et al 2020). Specific fungi that infect wood are important for nest-making in some cavity dwelling birds (Jusino et al 2015). Caterpillars that infest trees nourish migrating birds (Strode 2009, Hinkes et al 2015). Therefore, it is important to assess impacts of Darling 58 on the forest ecosystem from this non-agricultural perspective.

The widespread presence of OxO reported in various plant taxa implies similarly widespread presence of plant pathogens that use oxalic acid to kill plant cells during infection (e.g. Petition at 54-56, Ilyas et al 2016, Moosa et al 2017). Production of oxalic acid also has functions besides facilitating pathogenicity, and OxO has other functions as well (e.g. Petition at 52-53, 59, 62-63; Lou and Baldwin 2006, Mao et al 2007). Impacts to organisms other than *C. parasitica* in forests of eastern North America that produce oxalic acid and could be affected by the constitutively high levels of OxO made by Darling 58, such as in root exudates or in nuts and seedlings, must be considered.

In addition to a wheat OxO gene, Darling 58 is engineered with an antibiotic resistance gene, NPTII, from the bacterium *Escherichia coli* (Petition at 87). Environmental and health risks associated with expression of this selectable marker gene in Darling 58 growing in forest ecosystems have not been addressed (Chen et al 2012).

### Impacts of Darling 58 on other plants

There are two species of *Castanea* native to North America, the American chestnut and chinquapin (split into two species by some) (Petition at 25-26). Other chestnut species have been introduced from Asia and Europe mainly for horticulture. Hybrids and grafted trees have been produced in different *Castanea* species combinations for nut production in North America, and in an attempt to improve disease resistance (Petition at 29-30; “American chestnut can outcross to other chestnut species, including Chinese chestnut (*C. mollissima*), Japanese chestnut (*C. crenata*), European chestnut (*C. sativa*), and chinquapin (*C. pumila*) (Jaynes, 1964) to form hybrids (Section 2.1.2)”, Petition at 33; Craddock and Perkins 2019 at 126-127, Pereira-Lorenzo et al 2019).

If deregulated, without patent protection and with plans to distribute early Darling 58 lines immediately, anyone who is so inclined can and will attempt all types of crosses (“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,” Petition at 5; “Darling 58 also could be used to breed with other *Castanea* species (Section 2.1), such as European chestnut (*Castanea sativa*) and North American chinquapin species (*C. pumila* and *C. ozarkensis*), all of which are susceptible to chestnut blight.” Petition at 20).

Thus, in the short-term humans will be the most likely pollinators and propagators of Darling 58 and offspring and will greatly increase the speed and range of dispersal relative to unaided dispersal. There will be no record-keeping requirements. Nuts will be sent in the mail, scion wood for grafts will be passed around. In many cases, people who made the crosses and grafts will move away or die and the parentage of trees left behind will be forgotten. Impacts of Darling 58 will thus be difficult to study or mitigate post-deregulation because of this chaotic and ultimately untraceable distribution scenario (Woodcock et al 2018).

The Petition claims that genetic diversity of wild trees will be maintained because Darling 58 will be hemizygous for the OxO gene, always resulting in some progeny that are “fully wild-

type.” (“It is intended to outcross with wild relatives, which would allow the transgene to introgress into wild populations. It was intentionally not developed in a homozygous transgenic state, which will allow enhanced diversity via outcrossing and continual production of fully wild-type offspring.” Petition at 184; and, “this presence of non-transgenic offspring will be a useful conservation tool that allows production of the original wild-type trees long into the foreseeable future.” Petition at 81). This scenario does not account for OxO-homozygous trees that will be produced as offspring of Darling 58 mate in nature; or for the evolutionary fitness of various genetic combinations, including hybrids with compatible species (theoretical considerations: Renaut and Reiseberg 2015, Gaut et al 2015; hybrids: Hoban et al 2012). American chestnut trees are still living mainly in the understory throughout its range, flowering under some conditions (e.g. Laport 2020, Laport et al 2020, McNab 2017, Stephenson et al 1991) APHIS must consider impacts on *Castanea* populations under different natural selection scenarios.

In addition, new GE American chestnut “events” will be developed and released as part of the restoration breeding program (“We intend to develop at least one additional transgenic founder by inserting OxO into different American chestnut backgrounds than Ellis1, and expressing OxO using a wound-inducible promoter rather than the CaMV 35S promoter. Our primary purpose in creating additional founder(s) is to mitigate the risks of inbreeding depression from transgene-linked alleles and transgene silencing”, Westbrook et al 2020 at 92; “We currently plan to create up to three additional transgenic founders through *Agrobacterium*-mediated insertion of OxO into three American chestnut trees' genomes. Creating more transgenic founders will alleviate the founder bottleneck on effective population size once transgenic founders are outcrossed to WT trees”, Westbrook et al 2020 at 86).

As we understand it, currently regulated American chestnut trees with OxO genes – “legacy events” – will be automatically exempt from regulation under new Part 340 rules if this Petition is granted, as will future transformation events with the same mechanism of action (Part 340.1 (c), Federal Register/Vol. 85, No. 96/Monday, May 18, 2020/Rules and Regulations 29832-33<sup>1</sup>), contrary to the statement in Petition indicating further regulatory actions would be taken

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<sup>1</sup> Federal Register/Vol. 85, No. 96/Monday, May 18, 2020/Rules and Regulations 29832-33

## **PART 340—MOVEMENT OF ORGANISMS MODIFIED OR PRODUCED THROUGH GENETIC ENGINEERING**

### **§ 340.1 Applicability of this part.**

(a) The regulations in this part apply to those organisms described in § 340.2, but not to any organism that is exempt from this part under paragraph (b), (c), or (d) of this section.

...

(c) The regulations in this part do not apply to a plant with:

(1) A plant-trait-mechanism of action combination that has previously undergone an analysis by APHIS in accordance with § 340.4 and has been determined by APHIS not to be regulated under this part, or

(2) A plant-trait-mechanism of action combination found in a plant that APHIS determined to be deregulated in response to a petition submitted prior to October 1, 2021, pursuant to § 340.6 as that section was set forth prior to August 17, 2020. All plants determined by APHIS to be deregulated pursuant to § 340.6 as that section was set forth prior to August 17, 2020 will retain their nonregulated status under these regulations.

(“Additional events beyond Darling 58 may be produced and submitted for evaluation by APHIS in the future to further increase genetic diversity (especially alleles nearby the transgene insertion site) during the diversification breeding.” Petition at 112, underlining is ours). APHIS should clarify whether these GE trees and future ones will be regulated under the new Part 340 regulations, and if so, describe the nature of that regulation.

All of these new and “legacy” OxO-engineered American chestnut trees will join the gene pool that includes Darling 58. Potential impacts of intentional or natural “stacking” of various OxO events must be considered, including increases in OxO levels with more gene copies and different regulatory elements that increase non-target exposure, or conversely, gene silencing that may reduce tolerance to blight.

### Impacts of Darling 58 on animals

Very many animal species in a variety of taxa will interact with Darling 58 trees whether planted for horticulture or ecological restoration (e.g. Petition at 18, 32-42).

Direct impacts on animals arise mainly from consuming Darling 58 pollen, nuts, leaves and other parts of the tree. Unlike plants that naturally produce OxO, Darling 58 has high levels throughout the plant (“Most of the above examples of endogenous OxO activity involve temporary, localized, or induced OxO expression. This is presumably because overexpressing a defense response typically has metabolic costs (Karasov *et al.*, 2017), resulting in selection pressures for optimal (minimally functional) expression levels.” Petition at 60). Some animals interacting with Darling 58 are thus likely to be exposed to higher OxO concentrations, or to OxO for the first time. Differences in other chemical components in Darling 58 tissues and organs arising from random, event-specific mutations and epigenetic changes need to be considered, as well (e.g. Sala Junior et al 2008).

It is important for APHIS to base its assessments on Darling 58-specific information. Given the young age of Darling 58 trees and corresponding dearth of tissue samples, conclusions from most of the animal experiments described in the Petition are too preliminary to depend upon.

For example, impacts of Darling 58 on pollinators are important to assess (“American chestnut provided a food source to numerous insect species, especially during the flowering period.” Petition at 37). Petitioners did experiments to study how bumble bees might be affected by Darling 58, but did not have enough Darling 58 pollen for the experiments so used non-transgenic pollen instead, to which they added purified OxO from barley seeds (“Due to limitations on pollen production by transgenic trees, purified barley OxO enzyme (Roche Diagnostics, Mannheim, Germany) was added to non-transgenic chestnut pollen for this experiment.” Petition at 138).

Interpretation of results is thus complicated by not knowing how much OxO is actually present in Darling 58 pollen due to insufficient material. (“Real-world exposure of pollinators to OxO depends on transgene expression in pollen, which was not feasible to measure in currently available quantities of transgenic pollen.” Petition at 138).

Other important initial studies on animals reported in the Petition are of limited use because they involved feeding leaves from the Darling 4 instead of Darling 58 (tadpole studies, Petition at 179-181; and the aquatic insect herbivory study with caddisfly larvae, Petition at 176-178), even though Darling 4 has much lower levels of OxO in leaves (Petition at 96), again invalidating the conclusions for risk assessments.

Indirect impacts on animals include changes in habitat that will result as Darling 58 trees replace other trees in forests, including shifting dynamics of predator and prey populations from availability of different food sources, pest and pathogen pressures, and other cascading effects over time. Climate change will greatly influence all of these interactions (Petition at 40-41, Barnes and Delborne 2019).

### Impacts of Darling 58 on humans

People will mainly be exposed to Darling 58 through eating nuts and breathing in pollen.

OxO is commonly found in many foods, particularly grains (Petition at 126). However, Darling 58 has much higher levels in nuts than grains do (Petition at 127), and consequences for health must be considered.

Transgene products and other components have not yet been studied in pollen from Darling 58 yet (Petition at 127-128). Although the promoter used to regulate expression of the OxO transgene in Darling 58 usually results in low pollen expression in other plants, sometimes particular transformation events do express well in pollen (Petition at 139). Darling 58 pollen must be empirically tested for differences that could increase allergenicity in order to assess this risk.

### Environmental Impact Statement

If APHIS does not outright deny the petition, an Environmental Impact Statement is required to assess impacts of Darling 58 deregulation because the proposed uses of this tree will significantly affect the environment. This easily surpasses the low bar of any action that raises significant questions or that might significantly affect the environment. This is also the first ever GE tree of this type, setting the precedent for regulation (or complete lack thereof) of future approvals. An EIS is important for trees engineered to be released into the wild. (“USDA only considers forest health issues in the Plant Pest Risk Assessment if those issues are also “plant pest” issues, such as weediness or impacts on nontarget organisms. USDA may consider some forest health issues in its compliance with NEPA, but that analysis is much more likely when USDA conducts an EIS and not the less burdensome EA.”, NASEM at 152).

The EIS must include direct, indirect and cumulative effects of impacts outlined above and throughout these comments, but also of other issues related to environmental, social, cultural, health, and economic impacts, and endangered species. APHIS also needs to consider all relevant treaties, intergovernmental agreements, and international mandates. For example, with projected climate change the range of American chestnut will encompass more of Canada (Barnes and Delborne 2020, Petition at 23). The deregulation decision also requires direct participation and

full consent of Native American tribes and landholders impacted by Darling 58 throughout the historical and projected range of the American chestnut (Petition at 190; Barnhill-Dilling et al 2020).

#### Assessment of risks and impacts using the best available information from many sources

In order to make the best assessments, APHIS must critically analyze information from a variety of sources. Whenever possible, APHIS should consult high-quality independent peer-reviewed research, up-to-date reports in the press and extension bulletins, government studies, and other sources of relevant information. APHIS must also consult with all other federal and state agencies with relevant expertise, such as the US Fish and Wildlife Service.

#### References cited

Bagchi, R., Gallery, R.E., Gripenberg, S., Gurr, S.J., Narayan, L., Addis, C.E., Freckleton, R.P. and Lewis, O.T. (2014). Pathogens and insect herbivores drive rainforest plant diversity and composition. *Nature*, 506(7486), 85-88.

Barnhill-Dilling, S. K., Rivers, L., & Delborne, J. A. (2020). Rooted in Recognition: Indigenous Environmental Justice and the Genetically Engineered American Chestnut Tree. *Society & Natural Resources*, 33(1), 83-100.

Barnes, J. C., & Delborne, J. A. (2019). Rethinking restoration targets for American chestnut using species distribution modeling. *Biodiversity and Conservation*, 28(12), 3199-3220.

Bever, J. D., Mangan, S. A., & Alexander, H. M. (2015). Maintenance of plant species diversity by pathogens. *Annual review of ecology, evolution, and systematics*, 46, 305-325.

Boland, G. J., Ambrose, J., Husband, B., Elliott, K. A., & Melzer, M. S. (2012). Recovery Strategy for the American Chestnut (*Castanea dentata*) in Ontario (Ontario Recovery Strategy Series). Prepared for the Ontario Ministry of Natural Resources.  
[https://files.ontario.ca/environment-and-energy/species-at-risk/stdprod\\_075550.pdf](https://files.ontario.ca/environment-and-energy/species-at-risk/stdprod_075550.pdf)

Brienen, R.J.W., Caldwell, L., Duchesne, L., Voelker, S., Barichivich, J., Baliva, M., Ceccantini, G., Di Filippo, A., Helama, S., Locosselli, G.M. and Lopez, L. (2020). Forest carbon sink neutralized by pervasive growth-lifespan trade-offs. *Nature Communications*, 11(1), pp.1-10.

Chen, J., Jin, M., Qiu, Z.-G., Guo, C., Chen, Z.-L., Shen, Z.-Q., Wang, X.-W., & Li, J.-W. (2012). A survey of drug resistance bla genes originating from synthetic plasmid vectors in six Chinese rivers. *Environmental Science & Technology*, 46(24), 13448–13454.  
<https://doi.org/10.1021/es302760s>

- Clark, S. L., Schlarbaum, S. E., Pinchot, C. C., Anagnostakis, S. L., Saunders, M. R., Thomas-Van Gundy, M., Schaberg, P., McKenna, J., Bard, J. F., Berrang, P. C., Casey, D. M., Casey, C. E., Crane, B., Jackson, B. D., Kochenderfer, J. D., Lewis, R. F., MacFarlane, R., Makowski, R., Miller, M. D., Rodrigue J.A., Stelick J., Thornton C.D., & Williamson, T. S. (2014). Reintroduction of American Chestnut in the National Forest System. *Journal of Forestry*, **112**(5), 502–512. <https://doi.org/10.5849/jof.13-106>
- Craddock, J. H., & Perkins, M. T. (2019). Chestnut (*Castanea* spp. Miller) Breeding. In *Advances in Plant Breeding Strategies: Nut and Beverage Crops* (pp. 105-156). Springer, Cham.
- Dalgleish, H. J., Nelson, C. D., Scrivani, J. A., & Jacobs, D. F. (2016). Consequences of Shifts in Abundance and Distribution of American Chestnut for Restoration of a Foundation Forest Tree. *Forests*, **7**(1), 4. <https://doi.org/10.3390/f7010004>
- Gaut, B. S., Díez, C. M., & Morrell, P. L. (2015). Genomics and the contrasting dynamics of annual and perennial domestication. *Trends in Genetics*, **31**(12), 709-719.
- Hinks, A. E., Cole, E. F., Daniels, K. J., Wilkin, T. A., Nakagawa, S., & Sheldon, B. C. (2015). Scale-dependent phenological synchrony between songbirds and their caterpillar food source. *The American Naturalist*, **186**(1), 84-97.
- Hoban, S. M., McCleary, T. S., Schlarbaum, S. E., Anagnostakis, S. L., & Romero-Severson, J. (2012). Human-impacted landscapes facilitate hybridization between a native and an introduced tree. *Evolutionary Applications*, **5**(7), 720-731.
- Ilyas, M., Rasheed, A., & Mahmood, T. (2016). Functional characterization of germin and germin-like protein genes in various plant species using transgenic approaches. *Biotechnology Letters*, **38**(9), 1405–1421. <https://doi.org/10.1007/s10529-016-2129-9>
- Jia, S., Wang, X., Yuan, Z., Lin, F., Ye, J., Lin, G., Hao, Z. and Bagchi, R. (2020). Tree species traits affect which natural enemies drive the Janzen-Connell effect in a temperate forest. *Nature communications*, **11**(1),1-9.
- Jusino, M. A., Lindner, D. L., Banik, M. T., & Walters, J. R. (2015). Heart rot hotel: fungal communities in red-cockaded woodpecker excavations. *Fungal Ecology*, **14**, 33-43.
- Kolp, M., Double, M. L., Fulbright, D. W., MacDonald, W. L., & Jarosz, A. M. (2020). Spatial and temporal dynamics of the fungal community of chestnut blight cankers on American chestnut (*Castanea dentata*) in Michigan and Wisconsin. *Fungal Ecology*, **45**, 100925.
- Laport, R. G. (2016). Remnant American Chestnut (*Castanea dentata* (Marsh.) Borkh.; Fagaceae) in Upland Forests of Northwestern New York. *bioRxiv*, 068544.
- Laport, R. G., Smith, D., & Ng, J. (2020). Remnant American Chestnut (*Castanea dentata*) Near the Historical Western Range Limit in Southwestern Tennessee. *Castanea*, **85**(2), 232-243.

- Latham, J. R., Wilson, A. K., & Steinbrecher, R. A. (2006). The mutational consequences of plant transformation. *Journal of Biomedicine and Biotechnology*, 2006.
- Lindenmayer, D. B., & Laurance, W. F. (2016). The unique challenges of conserving large old trees. *Trends in Ecology & Evolution*, 31(6), 416-418.
- Lindenmayer, D. B., & Laurance, W. F. (2017). The ecology, distribution, conservation and management of large old trees. *Biological Reviews*, 92(3), 1434-1458.
- Lou, Y., & Baldwin, I. T. (2006). Silencing of a germin-like gene in *Nicotiana attenuata* improves performance of native herbivores. *Plant Physiology*, 140(3), 1126-1136.
- Lovat, C.-A., & Donnelly, D. J. (2019). Mechanisms and metabolomics of the host–pathogen interactions between Chestnut (*Castanea* species) and Chestnut blight (*Cryphonectria parasitica*). *Forest Pathology*, 49(6), e12562. <https://doi.org/10.1111/efp.12562>
- Mao, J., Burt, A. J., Ramputh, A.-I., Simmonds, J., Cass, L., Hubbard, K., Miller, S., Altosaar, I., & Arnason, J. T. (2007). Diverted Secondary Metabolism and Improved Resistance to European Corn Borer (*Ostrinia nubilalis*) in Maize (*Zea mays* L.) Transformed with Wheat Oxalate Oxidase. *Journal of Agricultural and Food Chemistry*, 55(7), 2582–2589. <https://doi.org/10.1021/jf063030f>
- Mcnab, W. H. (2017). Evidence that *Castanea dentata* Persists on Xeric, Mid-Elevation Sites in the Southern Appalachians. *Journal of North Carolina Academy of Science*, 133(1), 23-31.
- Miguel, C. and Marum, L. (2011). An epigenetic view of plant cells cultured *in vitro*: somaclonal variation and beyond. *Journal of Experimental Botany* 62, 3713–3725.
- Moosa, A., Farzand, A., Sahi, S. T., & Khan, S. A. (2017). Transgenic expression of antifungal pathogenesis-related proteins against phytopathogenic fungi – 15 years of success. *Israel Journal of Plant Sciences*, 65(01–02), 38–54.
- NASEM (2019). National Academies of Sciences, Engineering, and Medicine 2019. *Forest Health and Biotechnology: Possibilities and Considerations*. Washington, DC: The National Academies Press. <https://doi.org/10.17226/25221>.
- Neelakandan, A.K., and Wang, K. (2012). Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. *Plant Cell Reports* 31, 597 – 620.
- Nelson, C.D., Powell, W.A., Merkle, S.A., Carlson, J.E., Heberd, F.V., Islam-Faridi, N., Staton, M.E., & Georgi, L. (2014). Biotechnology of Trees: Chestnut. In: Ramawat, K.G., Mérillon, J-M., & Ahuja, M.R. (Eds), *Tree Biotechnology*. CRC Press, Boca Raton, FL, 3 – 35.

Pereira-Lorenzo, S., Costa, R., Anagnostakis, S., Serdar, U., Yamamoto, T., Saito, T., Ramos-Cabrer, A.M., Ling, Q., Barreneche, T., Robin, C. and Botta, R. (2016). Interspecific hybridization of chestnut. *Polyploidy and Hybridization for Crop Improvement*. CRC Press, Boca Raton, pp.377-407.

Renaut, S., & Rieseberg, L. H. (2015). The accumulation of deleterious mutations as a consequence of domestication and improvement in sunflowers and other compositae crops. *Molecular Biology and Evolution*, 32(9), 2273-2283.

Russin, J. S., Shain, L., & Nordin, G. L. (1984). Insects as Carriers of Virulent and Cytoplasmic Hypovirulent Isolates of the Chestnut Blight Fungus. *Journal of Economic Entomology*, 77(4), 838–846. <https://doi.org/10.1093/jee/77.4.838>

Sala Junior, V., Celloto, V. R., Vieira, L. G. E., Gonçalves, J. E., Gonçalves, R. A. C., & de Oliveira, A. J. B. (2008). Floral nectar chemical composition of floral nectar in conventional and transgenic sweet orange, *Citrus sinensis* (L.) Osbeck, expressing an antibacterial peptide. *Plant Systematics and Evolution*, 275(1-2), 1.

Stephenson, S. L., Adams, H. S., & Lipford, M. L. (1991). The present distribution of chestnut in the upland forest communities of Virginia. *Bulletin of the Torrey Botanical Club*, 24-32.

Strode, P. K. (2009). Spring tree species use by migrating yellow-rumped warblers in relation to phenology and food availability. *The Wilson Journal of Ornithology*, 121(3), 457-468.

Wang, G. G., Knapp, B. O., Clark, S. L., & Mudder, B. T. (2013). The Silvics of *Castanea dentata* (Marsh.) Borkh., American chestnut, Fagaceae (Beech Family). Gen. Tech. Rep. SRS-GTR-173. Asheville, NC: U.S. Department of Agriculture Forest Service, Southern Research Station. 18 p., 173, 1–18. [https://www.srs.fs.fed.us/pubs/gtr/gtr\\_srs173.pdf](https://www.srs.fs.fed.us/pubs/gtr/gtr_srs173.pdf)

Westbrook, J. W., Holliday, J. A., Newhouse, A. E., & Powell, W. A. (2020). A plan to diversify a transgenic blight-tolerant American chestnut population using citizen science. *Plants, People, Planet*, 2(1), 84–95. <https://doi.org/10.1002/ppp3.10061>

Wilson, A. K., Latham, J. R., & Steinbrecher, R. A. (2006). Transformation-induced mutations in transgenic plants: analysis and biosafety implications. *Biotechnology and Genetic Engineering Reviews*, 23(1), 209-238.

Woodcock, P., Cottrell, J. E., Buggs, R. J. A., & Quine, C. P. (2018). Mitigating pest and pathogen impacts using resistant trees: A framework and overview to inform development and deployment in Europe and North America. *Forestry: An International Journal of Forest Research*, 91(1), 1–16. <https://doi.org/10.1093/forestry/cpx031>