Submission to USDA APHIS docket APHIS-2020-0030: Petition for Determination of Nonregulated Status for Blight-Tolerant Darling 58 American Chestnut (*Castanea dentata*)

October 2020

Allison Wilson, PhD
Science Director
The Bioscience Resource Project
https://biosciencesource.org/

This response refers to the **Petition for Determination of Nonregulated status for blight- tolerant Darling 58 American Chestnut**, document number APHIS-2020-0030-0002, available at: https://www.regulations.gov/document?D=APHIS-2020-0030-0002 and referred to in this comment as "the Petition".

This comment will focus on some of the specific defects of the genetically engineered (GE) Darling 58 Chestnut, as well as some of the deficiencies in the scientific analysis of the Darling 58 Chestnut event/line and in the data provided in document number APHIS-2020-0030-0002.

Molecular Genetic Analysis of Darling 58

The use of genetic engineering as a plant-breeding tool results in numerous unintended, and frequently detrimental or harmful, molecular and phenotypic effects. This tendency is well documented in the scientific literature and in various scientific reviews (e.g. reviewed in Cellini et al. 2004, Wilson et al. 2014, Wilson et al. 2016 and Wilson 2020, *in press; See also The Nature Institute: Unintended Effects of Genetic Manipulation Project*). Thus careful molecular genetic characterization of every independent transgenic event or line is essential to prevent commercialization of poor quality or hazardous GE plants or crops (see Wilson 2020 for specific examples and a case study of GE Golden Rice). Genomic mutations (including deletions, insertions, point mutations, and rearrangements such as inversions and duplications) can occur at the site of transgene insertion and throughout the genome and these can have adverse phenotypic consequences (e.g. Wilson et al. 2014, Wilson et al. 2016 and Wilson 2020). The presence of such transformation-induced mutations and/or the presence of certain transgene components (such as the CaMV or the nos terminator) increase the likelihood of unintended and potentially detrimental effects or traits in a transgenic plant.

Darling 58 transgenic American Chestnut has been genetically engineered to express a transgene specifying an enzyme called oxalate oxidase (OxO). The expression of OxO in Darling 58 Chestnut is intended to confer tolerance to the fungal pathogen, *Cryphonectria parasitica*. To produce Darling 58, two transgenes were inserted into the

Ellis American Chestnut recipient line using *Agrobacterium* transformation of "somatic embryo clumps" (p.75). The two transgenes were: p35S-OxO-ActIII (a transgene specifying oxalate oxidase enzyme from wheat) and pUBQ10-NPTII-Nos, (a transgene specifying a selectable marker gene from bacteria that confers antibiotic resistance).

The first concern is the use of the CaMV (cauliflower mosaic virus) 35S promoter to regulate OxO. The CaMV promoter is a poor choice for various reasons. For example, it is prone to unintended effects and has been shown to be unstable under certain circumstances (e.g. Al-Kaff et al. 2000). The CaMV promoter also overlaps with viral Gene VI (Podevan and Du Jardin 2012) and this raises various safety concerns (Podevan and Du Jardin 2012, Latham and Wilson 2013a,b). For example, Podevin and Du Jardin concluded that the presence of segments of Gene VI *"might result in unintended phenotypic changes".* As noted by Latham and Wilson (2013a), *"They reached this conclusion because similar fragments of Gene VI have already been shown to be active on their own (e.g. De Tapia et al., 1993)."* Also, as discussed by Latham and Wilson (2013a) gene VI has been shown to act as an inhibitor of RNA silencing; to act as a transactivator of gene expression; to interfere with plant host defenses; and it is a potential toxin and/or allergen.

Furthermore, the CaMV 35S promoter has the potential to result in the mis-expression of adjacent genes. The Darling 58 petition claims (page 93): "*While the 35S promoter and associated enhancers have been shown to affect expression of nearby host genes in other plants (Wilson et al., 1996; Yoo et al., 2005; Gudynaite-Savitch et al., 2009), this effect is most commonly observed on genes within 3 kb of the 35S sequences and has not been reported to occur beyond 4.3 kb from 35S sequences (Weigel et al., 2000; Tani et al., 2004)*." The petition also states: "*The insert location in Darling 58 is more than 10.9 kilobases (kb) from the nearest upstream gene, as no known genes are present between the insert and the end of this scaffold based on current annotations. The nearest downstream gene is approximately 5.5 kb from the insertion site based on Augustus predictions (Stanke and Morgenstern, 2005)*". However (1) as mentioned in the petition, the American Chestnut genome and the Darling 58 genomes are still unfinished/incomplete, consequently, little is known about their structure/function. Thus additional functional sequences could be located near the transgene insert; (2) the actual distance between the OxO transgene and known neighboring genes in Darling 58 is still unknown and (3) other reviewers have noted that that "*Such promoters* [eg the CaMV 35S promoter] *have been shown to alter endogenous gene expression at a distance of up to 12 kbp (Wilson et al., 1996; Weigel et al., 2000; Jeong et al., 2002; Ichikawa et al., 2003)*" (see Wilson et al. 2006). Therefore specific experiments are necessary to look for the mis-regulation of other genes caused by the Darling 58

transgene, with a specific focus on gene sequences located within approximately 12kb from the CaMV 35S promoter. The data and arguments provided in the petition are not sufficient to dismiss mis-regulaton of endogenous genes.

A final reason to reject the use of the CaMV 35S promoter is the potential for horizontal gene transfer of viral transgenic inserts (such as CaMV 35S sequences) into viruses, creating novel viruses with novel functions (Latham and Steinbrecher 2004). Given that many other non-viral promoters are available for use in genetic engineering, the use of the CaMV 35S promoter in the OxO gene cassette was a mistaken choice and greatly increases the potential for harmful unintended impacts and unwanted traits in Darling 58. As the use of the CaMV 35S promoter in Darling 58 was totally unnecessary the choice to use it gives rise to completely unnecessary risks. In general, and in the specific case of Darling 58, the CaMV 35S promoter should not be considered an acceptable component of GE trees created for release into the wild and for use in breeding programs. It should also be noted that experiments to test for the presence of unintended effects arising from the use of the CaMV 35S promoter in Darling 58 have not been carried out.

Another defect of Darling 58 is the presence of the superfluous selectable marker gene pUBQ10-NPTII-Nos that confers antibiotic resistance. While marker genes can be useful during the initial stages of producing a genetically engineered organism, all superfluous DNA increases the potential for unintended effects (Wilson et al. 2004). The presence of superfluous DNA (such as selectable markers) in GE crops or trees (or other GE organisms) can disrupt metabolism or introduce unwanted traits. Furthermore, the use of antibiotic resistance genes raises the risk of horizontal gene transfer to soil bacteria or to the gut bacteria of organisms consuming pollen, nuts or leaves. Furthermore, the NPTII construct present in Darling 58 utilizes the nos terminator, which has long been known to be leaky. As noted by Wilson et al. (2006), "*transcriptional read-through and mRNA processing were shown to occur when the nos terminator was used in a transgene present in a commercially approved insertion event (Rang et al. 2005). In this case, the aberrant transcripts were processed into variants containing open reading frames (ORFs) which could give rise to chimaeric proteins.*" Such chimaeric proteins could result in a variety of unintended effects ranging from creating allergens or toxins to disrupting cellular metabolism. The presence of pUBQ10-NPTII-Nos in Darling 58 and the use of the nos promoter are both totally unnecessary and their presence gives rise to totally unnecessary risks. Neither marker genes nor nos promoters should be present in GE trees that are approved for growth in the wild or for breeding programs.

Finally it should be noted that D58 chestnuts have an approximately 600bp inversion at the transgene insertion site. Again this makes Darling 58 a poor choice of transgenic event and a likely candidate for unintended effects and unwanted traits (Wilson et al. 2004, 2006 and Wilson 2020).

Finally, the Petition for the D58 chestnut is missing the whole genome sequence and analysis necessary to complete the molecular genetic analysis of Darling 58. Needed are (1) the completed whole genome sequence for both the parental Ellis American Chestnut line and the Darling 58 transgenic American Chestnut line and (2) whole genome comparisons between them. A whole genome comparison between D58 and its parent Ellis is necessary to reveal whether there are additional transformation-induced mutations in the D58 genome, such as large scale rearrangements or small fragments of transgene, marker or plasmid DNA or other contaminating bacterial sequences, which would not be apparent without whole genome analysis (Wilson et al. 2006, Wilson et al. 2004).

However, given the defects apparent in D58 already discussed, even without a whole genome sequence, the limited molecular genetic analysis available makes it is clear that D58 in not an appropriate candidate for release into the wild or use in chestnut breeding programs and the petition for deregulation should be rejected.

Phenotypic analysis of GE Darling 58 American Chestnut

The experimental data on Darling 58 submitted in the petition for deregulation should be adequate to ensure:

1. The efficacy and durability of the blight tolerance trait specified by the OxO transgene in D58, throughout its lifetime, in a forest or food crop setting.
2. The absence of unintended and detrimental characteristics in D58, throughout its lifetime, in a forest or food crop setting. This requires an assessment of traits such as growth and reproduction, pathogen resistance, volatiles, root exudates, nutritional quality and food safety of nuts, wood quality, impacts of D58 on forest or agri-forestry ecosystems – including impacts on biodiversity, soil health, and waterways. Assessment should also include metabolic, transcriptomic and proteomic comparisons between D58 and its parental line Ellis. Such studies would aid in the identification of harmful unintended effects.

However, the Petition states that the oldest Darling 58 trees alive at the time of writing are about 3 years old (p.77), and (p.144) that, "*No Darling 58 trees are yet mature enough to produce female flowers*...". This means that any long-term properties of GE

Darling 58 trees are completely unknown, as no tests such as those mentioned above can have been carried out on trees older than 3 years.

Furthermore, regarding tests that have been carried out, there is very limited testing material available due to the small number of trees and their limited age (leading to a lack of pollen and chestnuts for testing), and this has affected the statistical power of any studies and/or led to reliance on 'legacy events' which may not give the same results. This is true even when the 'legacy events' have identical transgenes to Darling 58, as it is well known and documented that every transgenic event must be tested individually and *in vivo* (because every independently derived transgenic event and line is unique) (Arpaia et al 2017, Wilson 2020, Wilson et al. 2006). With the absence of data on D58 trees that are older than 3 years, there is an enormous gap in the data needed to determine either (1) efficacy and durability or (2) the unintended effects/traits of D58, a gap that spans most of the potential 100 year or more lifetime of an American Chestnut, including its reproductive years.

Other Examples of Absent or Incomplete Data Discussed in the D58 Petition

First, no –OMIC data appear to have been provided. Such data could help identify aberrant proteins or transcripts generated by the genetic engineering process. It could also help identify any unintended metabolic effects resulting from the expression of the transgene and marker genes.

Next, as noted by GeneWatch UK in their Comment submitted September 2020: the Darling 58 petition itself states in numerous places that data for D58 are not yet available or the experiments submitted in the petition have low statistical resolution. Thus all early data may be invalid and/or incomplete. For example:

a. "*Tadpole development and survival*" is highlighted as untested for Darling 58 GE trees (Table 1.3a, p.21).

b. "*Additional pollinations with T1 pollen were performed in 2019; inheritance results will be published and/or shared when they are available (testing underway; results anticipated late spring 2020)*". (p.83)

c. "*More detailed genome analyses from Darling 58 and offspring will be shared as they become available (anticipated by late 2020).*" (p.88)

d. "*A preliminary insert map showing part of Chromosome 7 is shown in Appendix*

III; further details will be provided when they become available..." (p.92)

e. "*The American chestnut genome is still in draft form and has not yet been annotated, so comparisons to native genes are based on the Chinese chestnut genome*..." (p.92)

f. "*According to PCR and limited sequencing data, when Darling 58 sequences are compared to Ellis 1 genomic DNA, Darling 58 has an inversion of approximately 600 base pairs as shown in Figure 7.3.2c, just outside the left border. This inversion is not near any known genes (see above in this subsection). A more complete understanding of the genome sequence near the insertion site should be elucidated by a whole genome sequence of Darling 58 and offspring, which should be available soon as described above*." (p.94)

g. The "T1 Nut" samples that have been tested for oxalate oxidase quantities are from transgenic nuts from different mother trees (Figure 7.4.2a, p.100): there are no samples of chestnuts from Darling 58 GE mother trees because there are as yet no female flowers from such trees (as noted on p.144).

h. "*We have used several tests to assess blight tolerance on various chestnut tissues and trees, depending on the age and size of material available. This section describes intentional inoculations on Darling 58 tissues and trees using the chestnut blight pathogen Cryphonectria parasitica. Results of inoculations and natural blight infections on older OxO-expressing transgenic chestnut events are described in Section 10.5.1. Further tests on additional outcross generations of Darling 58 offspring will be performed when these trees are large enough to inoculate, and we will continue to share and/or publish results as they become available*." (p.101)

i. "*The small number of seedlings available for this inoculation limits statistical power*..." (p.103)

j. "*Due to limitations on numbers of available plants, growth rate of tissue culture-generated plants, and the size of field plots, quantitative measurements comparing growth rates and photosynthetic performance of transgenic vs. non-*

transgenic American chestnut trees have been limited. The most recent available measurements (Section 8.2.2) are from Darling 58 seedling offspring germinated spring 2019; this is the first year a large sample size (> 10 transgenic and non-transgenic seedlings) of Darling 58 seedling offspring has been available for measurement. However, first- season measurements of chestnut seedling height should be considered preliminary as they are not necessarily indicative of future growth, and may be more closely correlated to nut weight, family background, cultural treatments, or other factors". (p.107)

k. "*...data and conclusions should be considered preliminary until measurements can be conducted on older seedlings in controlled experimental plots*..." (p109)

l. "*We recognize that these analyses reflect a small number of measurements on a limited number of trees, and that they do not include other non-transgenic American chestnut types that would help put the results in context of natural variation. We also recognize that there could be biological effects of transgene insertion or expression on the photosynthetic and respiratory rates that we were unable to detect here, that such biological effects would only manifest at particular times of year or in particular growth conditions, that any of these effects might be due to linked endogenous chestnut genes near the insertion site rather than the insertion itself, or that such effects may be smaller than those caused by traditional breeding or other treatments. Finally, we have an ongoing effort to more fully characterize the photosynthetic and respiratory physiology of these trees (and others) in three common gardens across a climate gradient that will progress over the next few years (see BRAG project description, Section 11.2); results will be published and/or shared as they become available*." (p.119)

m. "*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*. *Studies on other transgenic plants suggest that transgene expression controlled by the 35S promoter is negligible in pollen, or expressed at a lower rate than vegetative tissues (see below in this section). 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." (p.138)

n. "...*many more years of research will be required to produce data about interspecific hybridization of Darling 58 and compatible species*..." (p.144)

o. "*Whole genome sequencing is in progress for Darling 4 and the isogenic line WB275- 27, which should further clarify details regarding insert location, copy number, structure, etc. Results will be shared when they are available (anticipated in 2020)*". (p.162)

p. Long-term research is planned after nonregulated status is granted (p.186)

q. Regarding the spread and establishment of chestnut trees, "*Each of these sources has a high degree of uncertainty due to the limited locations or data available, and establishment may be faster on areas with site conditions particularly favorable to chestnut recruitment*". (p.193)

r. "*Further sequence analysis of Darling 58 and transgenic offspring is underway; results will be published and/or shared when they become available*". (p. 236)

The poor quality or unfinished nature of the experiments submitted in the D58 petition, coupled with a complete absence of –OMIC analyses and of data on GE Darling 58 chestnuts older than 3 years of age, require the rejection of the D58 GE chestnut for deregulation.

Key Factors Unaddressed in the D58 Petition for Deregulation

As documented by GeneWatch (Sept 2020 comment) and/or Smolker and Petermann (2019), the deregulation and subsequent use of the Darling 58 event or lines in field/forest settings would pose extreme hazards to forest health and especially to remaining American Chestnuts – both surviving trees and sprouting stumps. These key facters include (1) there is inadequate data presented in the petition to show that adult Darling 58 Chestnuts would indeed be blight tolerant (the oldest living tree being approx. 3 years old) or (2) that if the trees were blight tolerant that the tolerance would be durable over the lifespan of the trees and under changing and varied environmental conditions; (3) Furthermore, if Darling 58 American Chestnuts were indeed blight tolerant they would inevitably act as a blight reservoir, further endangering native wild trees and sprouts and likely hastening the decline of all native American Chestnuts; (4). The unintended effects of the transgene (eg harmful metabolic impacts of the expression of either the intended OxO transgene or the superfluous NPTII selectable

marker gene) have not been adequately assessed for the available Darling 58 trees, but more importantly for Darling 58 trees of reproductive age and older. (5) Once Darling 58 was planted in the wild or crossed to native chestnuts it would become impossible to monitor its impacts due to cross-pollination and the lack of traceability. It would also, for these reasons, be impossible to "recall" Darling 58 as its hazards and failings became apparent over time. The lack of traceability would make food labeling (of nuts for human food or livestock) difficult or impossible.

As a further point, these key factors, and others detailed in the White paper by Smolker & Petermann (2019) and in the GeneWatch UK submitted comment (Sept 2020), highlight why a full EIS (Environmental Impact Statement) is necessary for the Darling 58 Chestnut.

Conclusion

As discussed in this comment, the intrinsic molecular shortcomings of the Darling 58 GE American Chestnut should trigger the rejection of the petition for deregulation. In addition, the most basic data and analysis necessary to assess efficacy of the intended transgene and to test whether it has potential harmful unintended impacts on humans, livestock, wildlife and wider ecosystems are either (a) still in progress (b) so limited as to be useless in making efficacy and safety determinations or (3) completely missing, as the oldest D58 chestnut is approx. 3 years old. Thus the actual quality and viability of D58 chestnuts in the long term, as well as the impacts of Darling 58 chestnuts in a forest or food system, cannot even begin to be assessed. Even if more data were made available later, as claimed in the petition, it would be too late for public comment or independent analysis of the data, and it would be impossible to recall the D58 event once in was deregulated and grown in the wild. Given the numerous technical weaknesses of the D58 event/line itself and the numerous scientific weaknesses of the D58 petition and its enormous data gaps, the **Petition for Determination of Nonregulated Status for Blight-Tolerant Darling 58 American Chestnut** should be denied.

REFERENCES

Note: These references are also attached as PDF files.

Al-Kaff, N. S., Kreike, M. M., Covey, S. N., Pitcher, R., Page, A. M., & Dale, P. J. (2000). Plants rendered herbicide-susceptible by cauliflower mosaic virus–elicited suppression of a 35S promoter-regulated transgene. *Nature biotechnology*, *18*(9), 995-999. Accessed October 2020 at: https://www.researchgate.net/profile/Nadia_Al-Kaff/publication/12349357_Plants_rendered_herbicide-susceptible_by_cauliflower_mosaic_virus-elicited_suppression_of_a_35S_promoter-regulated_transgene/links/53fa186d0cf2e3cbf5621f87.pdf

Arpaia, S., Birch, A. N. E., Kiss, J., Van Loon, J. J., Messéan, A., Nuti, M., ... & Tebbe, C. C. (2017). Assessing environmental impacts of genetically modified plants on non-target organisms: the relevance of in planta studies. *Science of the Total Environment*, *583*, 123-132. Accessed October 2020 at: https://www.researchgate.net/profile/Marco_Nuti/publication/313751145_Assessing_environmental_impacts_of_genetically_modified_plants_on_non-target_organisms_The_relevance_of_in_planta_studies_Salvatore_Arpaia_a_A_Nicholas_E_Birchb_Jozsef_Kiss_c_Joop_JA_v

Cellini, F., Chesson, A., Colquhoun, I., Constable, A., Davies, H. V., Engel, K. H., ... & Lehesranta, S. (2004). Unintended effects and their detection in genetically modified crops. *Food and Chemical Toxicology*, *42*(7), 1089-1125. Accessed October 2020 at: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.321.9358&rep=rep1&type=pdf

GeneWatch UK (September 2020) Submission to USDA APHIS docket APHIS-2020-0030: Petition for Determination of Nonregulated Status for Blight-Tolerant Darling 58 American Chestnut *Castanea dentata*) Accessed October 2020 at http://www.genewatch.org/uploads/f03c6d66a9b354535738483c1c3d49e4/genewatch-uk-aphis-ge-chestnut-fin.pdf.

Latham, J., & Wilson, A. (2013a) Regulators Discover a Hidden Viral Gene in Commercial GMO Crops. *Independent Science News* accessed October 2020 at: https://www.independentsciencenews.org/health/regulators-discover-a-hidden-viral-gene-in-commercial-gmo-crops/

Latham, J., & Steinbrecher, R. (2004) GM Gene Flow (B): Horizontal gene transfer of viral inserts from GM plants to viruses. *Econexus Technical Paper* Accessed on October 2020 at: https://www.econexus.info/sites/econexus/files/Horizontal-Genes-virus-2004.pdf

Latham, J., & Wilson, A. (2013b). Is the Hidden Viral Gene Safe? GMO Regulators Fail to Convince. *Independent Science News* accessed October 2020 at: https://www.independentsciencenews.org/health/gmo-regulators-hidden-viral-gene-vi-regulators-fail/

The Nature Institute, Unintended Effects of Genetic Manipulation Project (2009-2019). Website accessed October 2020: https://www.natureinstitute.org/nontarget

Podevin, N., & Du Jardin, P. (2012). Possible consequences of the overlap between the CaMV 35S promoter regions in plant transformation vectors used and the viral gene VI in transgenic plants. *GM crops & food*, *3*(4), 296-300. Assessed October 2020 at: https://www.tandfonline.com/doi/pdf/10.4161/gmcr.21406

Smolker, R., & Petermann, A. (2019). *Biotechnology for forest health? The Test Case of the Genetically Engineered American Chestnut.* Produced by The Campaign to STOP GE Trees, Biofuelwatch and Global Justice Ecology Project. Accessed October 2020 at: https://stopgetrees.org/wp-content/uploads/2019/04/biotechnology-for-forest-health-test-case-american-chestnut-report-WEB-1.pdf

Wilson, A. (2020, *in press*) "Will gene-edited and other GM crops fail sustainable food systems?" in Kassam, A., & Kassam, L. (Eds.). *Rethinking Food and Agriculture: New Ways Forward*, Elsevier, First Edition. Accessed on October 2020 at: https://inclusiveresponsibility.earth/ and https://www.elsevier.com/books/rethinking-food-and-agriculture/kassam/978-0-12-816410-5

Wilson, A., Latham, J., & Steinbrecher, R. (2004). Genome scrambling–myth or reality. *Transformation-induced mutations in transgenic crop plants (EcoNexus, Brighton, UK, 2004)*. Accessed October 2020 at: https://econexus.info/sites/econexus/files/ENx-Genome-Scrambling-Report.pdf

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. Accessed October 2020 at: https://www.tandfonline.com/doi/pdf/10.1080/02648725.2006.10648085