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Dr. Michael J. Firko
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Road, Unit 98
Riverdale, MD 20737

RECEIVED
By USDA APHIS BRS Document Control Officer at 2:53 pm, May 05, 2017

Re: Confirmation that transgene-free, genome edited tobacco plants are not regulated articles

Dear Dr. Firko,

My laboratory is applying genome-editing technologies toward the development of tobacco plants that produce very low levels of nicotine. Specifically, we are accomplishing this via targeted mutagenesis through the use of custom-designed meganuclease enzymes produced by the company Precision Biosciences that target a small gene family referred to as *Berberine Bridge Enzyme-Like (BBL)*. Genes of this family encode enzymes that catalyze one of the final steps of nicotine biosynthesis. The tobacco genome possesses six *BBL* genes, and RNAi-mediated suppression of this gene family has proven to be effective in conferring a low nicotine phenotype (Lewis et al., 2015, PLOS ONE 10(2):e0117273).

I. Transformation Method and Mutagenesis of Target Genes

Tobacco leaf discs were transformed using a disarmed *Agrobacterium tumefaciens* strain possessing a gene-editing nuclease within binary vector pCAMBIA2300, the individual components of which are described in detail below. Exposure of the tobacco cells to a custom-designed meganuclease targeting one or more members of the *BBL* family led to the production of genome-edited tobacco plants carrying small deletions and/or insertions at the targeted loci. The observed deletions ranged in size from 1 to 106 bp, and insertions of a maximum size of 47 bp were recovered. All observed insertion events represented short duplications of a nearby portion of the gene being targeted by the meganuclease; thus no foreign DNA sequences were introduced in any genome-edited *BBL* gene.

II. Method for Obtaining Null Segregants

Primary transformants were self-pollinated and the resulting progeny were screened by PCR for the presence or absence of the transgene using primers specific for the kanamycin resistance gene (*nptII*) and the meganuclease-encoding sequence. Further confirmation that PCR-negative individuals lacked sequences derived from the transformation construct was obtained by Southern blot analysis. It was possible to obtain transgene-free segregants with mutated *BBL* genes because the transgene constructs were not inserted in a chromosomal region closely linked to the target gene(s).

III. **Construct Components**

a. Mutation-Inducing Meganuclease Gene:

Promoter: 35S from Cauliflower Mosaic Caulimovirus – an enhanced 35S promoter from CaMV
Gene: Re-engineered meganuclease from *Chlamydomonas reinhardtii* – an *I-CreI* gene from the green algae *C. reinhardtii* was modified via directed evolution to produce an enzyme that would recognize and cut a specific sequence within tobacco *BBL* genes
Terminator: Nos from *Agrobacterium tumefaciens* – transcriptional termination sequences from the *Agrobacterium* nopaline synthase gene

b. **Selectable Marker Gene:**

Promoter: 35S from Cauliflower Mosaic Caulimovirus – an enhanced 35S promoter from CaMV
Gene: nptII from *Escherichia coli* – the neomycin phosphotransferase gene (*nptII*) that confers resistance to the antibiotic kanamycin
Terminator: 35S-t from Cauliflower Mosaic Caulimovirus – a termination and polyadenylation signal derived from CaMV

c. **Other Relevant Elements:**

RB and LB from *Agrobacterium tumefaciens* – the right and left T-DNA border sequences from the *Agrobacterium* Ti plasmid that facilitates insertion of the transgene into the recipient genome

IV. **Recipient Organism** – *Nicotiana tabacum*

Tobacco is a crop that is widely grown throughout the world. It is not a noxious weed or pest.

V. **Trait** – Reduced nicotine content

Inactivating genes of the *BBL* family through target mutagenesis reduces the capacity of the tobacco plant to produce nicotine. Maximal nicotine reduction is achieved as multiple *BBL* mutations are pyramided together within the same plant through normal breeding practices. There are several potential applications of low nicotine tobacco plants, including (1) the production of low-nicotine cigarettes as a smoking cessation tool to help wean smokers off of their nicotine habit; and (2) as a preferred platform when tobacco plants are used for the production of pharmaceuticals or as a biofuels feedstock.

Although certain components of the transgene used to facilitate targeted mutagenesis of *BBL* genes in the primary transformed lines possessed sequences derived from organisms classified as plant pests, the null segregants recovered in the next generation do not. It is my understanding that APHIS has made a number of determinations stating that null segregants are not considered regulated articles under 7 CFR § 340. Furthermore, tobacco is not a federal noxious weed or a plant pest. I therefore request that you confirm that the mutant *BBL* tobacco lines we have produced through genome editing, and that possess no

plant pest elements or any foreign DNA sequences, are not considered regulated articles by APHIS under current regulations.

I look forward to hearing your response to this letter of inquiry. Thank you for your consideration.

Sincerely,

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