February 29, 2016

Dr. Michael J. Firko
APHIS Deputy Administrator,
Biotechnology Regulatory Services
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Riverdale, MD 20737

DONALD DANFORTH
PLANT SCIENCE CENTER
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RECEIVED
By USDA APHIS BRS Document Control Officer at 1:58 pm, Mar 07, 2016

Re: Inquiry regarding APHIS position on null-segregant CRSPR-Cas9-mutagenized *Setaria viridis* lines as non-regulated articles.

Dear Dr. Firko,

With this letter, I am asking that Biotechnology Regulatory Services confirm my understanding that null segregant *Setaria viridis* plants (plants containing no transgenic sequences) derived from transgenic lines Cas9_193-16, Cas9_193-17, Cas9_193-24, and Cas9_193-31 do not meet the criteria for regulated articles.

Transformation method and resulting mutagenesis of the target gene
Transformation was achieved via disarmed *Agrobacterium tumefaciens*-mediated transformation. *Setaria viridis* genotype A10.1 was transformed with the construct described below. When integrated into the plant genome, the expressed CRISPR associated protein 9 (Cas9) was guided to a targeted sequence within the coding region of the *Setaria viridis* homolog of the *Zea mays* ID1 gene by the expressed guide RNAs. The Cas9 caused a double-stranded break in that site of the DNA, which when repaired by the recipient plant's endogenous DNA repair mechanisms, resulted in a mutation that deactivated the *S. viridis* ID1 homolog.

Method of obtaining null segregants
The original transformed plants were self-pollinated and the resulting offspring genotyped by PCR for the Cas9 junction and the hygromycin gene in the subsequent generation for individuals that were null segregants. This segregation was possible because the transgenic construct was not linked to the *S. viridis ID1* homolog. The null segregants now possess the deactivated *S. viridis ID1* homolog.

Construct

Gene(s) of Interest

Promoter: Ubiquitin from *Zea mays* - Maize ubiquitin promoter containing an intron sequence for strong constitutive expression in grass species.
Gene: CRISPR associated protein 9 (Cas9) from *Triticum aestivum* - Wheat codon-optimized Cas9 (CRISPR associated protein 9) is a RNA-guided DNA endonuclease enzyme to target a gene of interest sequence.

Terminator: NOS from *Agrobacterium tumefaciens* - Nopaline synthase terminator (NOS) used to end transcription of the Cas9 cassette.

Promoter: polymerase III promoter U6 from *Triticum aestivum* - Wheat polymerase III promoter (U6) used to drive expression of guide RNA of interest.
Gene: single guide RNA 36003-2 from *Setaria viridis* - Single guide RNA targeting the second exon of the *Setaria viridis* ID1 homolog (Sevir.9G247100). This is a homolog of the ID1 gene that promotes flowering in maize.
Terminator: OCS from *Agrobacterium tumefaciens* - Octopine synthase terminator (OCS) used to end transcription of the cassette.

Vector Sequence: RB from *Agrobacterium tumefaciens* - Right T-DNA border sequence from Ti plasmid for insertion into the plant genome.

Screenable Marker
Promoter: Ubiquitin from *Panicum virgatum* - Ubiquitin promoter from Panicum virgatum (PvUBI) for constitutive expression in grasses.
Gene: pporRFP from *Porites porites* - Red fluorescent protein marker (RFP) visual selection of transgenic plants.
Terminator: NOS from *Agrobacterium tumefaciens* - Nopaline synthase terminator used to stop gene transcription.

Selectable Marker
Vector Sequence: LB from *Agrobacterium tumefaciens* - Left T-DNA border sequence for integration into the plant genome.
Promoter: Ubiquitin2 from *Panicum virgatum* - Ubiquitin 2 promoter sequence from *Panicum virgatum* (PvUBI2) for constitutive expression in grasses.
Gene: HPT from *Escherichia coli* - Hygromycin phosphotransferase (HPT) gene for selection of transgenic plants.
Terminator: 35ST_polyA from Cauliflower mosaic caulimovirus - Terminator/polyadenylation signal from Cauliflower mosaic virus 35S.

Recipient organism – *Setaria viridis* genotype A10.1

Donor organisms
Zea mays
Triticum aestivum
Panicum virgatum
Setaria viridis
Porites porities
Escherichia coli
Cauliflower mosaic caulimovirus

Trait
Delayed flowering time

While the original transgenic line was produced with a construct containing plant pest sequences, the resulting null segregants do not contain such sequences. It is therefore my understanding that the null segregants are not regulated articles under 7 CFR Part 340. Please confirm that this understanding is correct. This letter contains no Confidential Business Information.

Sincerely,

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