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August 4, 2023
Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737
Re: Regulatory Status Review (RSR)

Confidential Business Information Deleted

Dear Ms. Juarez,

Bioheuris Inc. respectfully requests confirmation from the USDA-APHIS's Biotechnology Regulatory Services (BRS) regarding the regulatory status of a soybean (*Glycine max*) we intend to develop using CRISPR-Cas9 gene editing technology. The proposed soybean product contains two targeted base pair substitution (edits) in the protoporphyrinogen oxidase (*ppo*) gene[1] and is tolerant to herbicides that otherwise kill plants through inhibition of the essential PPO protein. Based on the data and information contained in the enclosed petition, we believe that the modified soybean plant does not present a plant pest risk and is not otherwise deleterious to the environment. The enclosed petition contains confidential business information. The undersigned certifies that, to the best of our knowledge and belief, this petition includes all data, information, and views relevant to the matter, whether favorable or unfavorable to the position of the undersigned, which is the subject of the petition.

As described below, our intended final plant line contains two targeted base pair substitutions in the same codon (edits) in the protoporphyrinogen oxidase (*ppo*) gene, resulting in one amino acid substitution (mutation) in the protein sequence. We request a regulatory status review under the new BRS SECURE rule published in 7 CFR part 340 section §340.4.

A. Requestor's name and contact information

Dr. Carlos Perez
Bioheuris Inc.
1100 Corporate Square Dr., St. Louis, MO 63132
Email: carlos.perez@bioheuris.com

B. Confidential Business Information (CBI) Statement

The enclosed petition contains confidential business information. A CBI Justification Statement will be provided.

C. Description of Comparator Plants

a. First Comparator Plant

Resistance to acetolactate synthase (ALS) and photosystem II inhibiting herbicides was confirmed in a population of allotetraploid annual bluegrass *(Poa annua L*) selected from golf course turf in Tennessee. Genetic sequencing revealed that seven of eight plants had a point mutation in the *psbA* gene resulting in a known S264G substitution and a homozygous A205F substitution on ALSb, caused by two nucleic acid substitutions in one codon. Whole plant testing confirmed that this substitution conferred resistance to simazine. This study shows that two nucleic acid substitutions in the same codon already occur in nature (Brosnan *et al.,* 2016). With this evidence we thought that the final product proposed in this consultation (T3 descendant transgenic-free, modified soybean) is comparable with the one isolated from golf course turf in Tennessee.

b. Second Comparator Plant

Dicamba resistance was first documented in kochia (Bassia scoparia) in 1994 in populations isolated from fields in western Nebraska and Montana. The Aux/IAA mutation identified from these natural populations of synthetic auxin resistant weed species was a double-nucleotide substitution in the IAA16 gene. The mutation consisted of the change from GGT (Gly) to AAT (Asn) at amino acid position 127 (G127N).This double mutation is located in the conserved degron of the Aux/IAA protein leading to low dicamba affinity in Aux/IAA protein complex (LeClere et al., 2018). This report shows evidence of a 2-nt base change in the one codon, comparable to the base edit we plan to introduce in the soybean HPPD gene.

c. Third Comparator Plant

Amaranthus hybridus L. is a competitive weed for summer crops in South America. In 2019 two independent research groups[2,3] report glyphosate resistant populations caused by a multiple amino acid substitutions in the *epsps* gene, known as TAP-IVS. The TAP-IVS substitutions consist of three aminoacid substitutions caused by 5 nucleotides changes in a 12 nucleotide region. The first mutation consisted of the change from ACA (Thr) to ATA (Ile) at amino acid position 102 (T102I). The second mutation consisted of the change from GCG (Ala) to GTC (Val) at amino acid position 103 (A103V). The third mutation consisted of the change from CCA (Pro) to TCA (Ser) at amino acid position 106 (P106S).

A. Genotype of the Modified Plant

a. Description of plant's genus, species

- Order: *Magnoliopsida*
- Family: *Fabaceae*
- Genus: *Glycine*
- Species: *Glycine max*

b. Description of reagents used for genome editing

The material is being developed using CRISPR/Cas gene editing technology, using a technique based on ribonucleoprotein (RNP) bombardment at the somatic embryo stage of a soybean in vitro culture system, followed by a regeneration protocol until obtaining seeds.

This technology is based on the use of nucleases that can be directed to their specific site in the genome by an RNA guide to make a precise and specific cut in the double-stranded DNA (DSB). When the cell detects the cut, it triggers the repair mechanisms, being able to introduce mutations during this process. In eukaryotes, DSB repair occurs mainly by two pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR). The NHEJ mechanism is prone to introduce errors during repair through insertions or deletions of some nucleotides (indels). In the mechanism called HDR (Homology Directed Repair), a DNA molecule is used as a template for DSB repair, allowing the precise introduction of the desired modification. Jinek et al., (2012) and Zetsche et al., (2015) have a detailed description of the mechanism of action of CRISPR-Cas [4,5]. Its application in plants requires the introduction of the nuclease, the guide, and the repair template into a cell from which the plant can regenerate. Zhang et al., (2018); Sharma et al., (2017), Ran et al. (2017) and Jaganathan et al., (2018) provide a detailed description of the gene editing scheme in plants[6–9], and examples of its application in maize and wheat can be found in Liang et al., (2017) and Svitashev et al., (2016), respectively[10,11].

The presence of the mutation will be confirmed by sequencing the target gene in the DNA of the regenerated plants. It is important to highlight that the product obtained by this strategy does not contain foreign DNA in the soybean genome and does not constitute a new combination of genetic material.

Steps involved to obtain the intended soybean plant:

1. Obtaining the embryonic axis from soybean seeds.
2. Design of a specific guide RNA (gRNA) to target the nuclease cleavage site at position [ ] (CDS) of the GmPPO2 gene [ ]. CBI Deleted
3. gRNA synthesis. The guide comes from chemical synthesis and does not involve stages in its production process in which it could be contaminated with DNA.
4. In-vitro tests of the efficiency and specificity of the nuclease cut.
5. Design and synthesis of the ssDNA repair template (BH1070).The repair template comes from chemical synthesis and its production does not involve steps in which DNA of another origin could be introduced. The length of the template sequence is 107 nucleotides and it contains the change to be introduced into the [ ] CBI Deleted gene. The repair template sequence contains a nucleotide change [ ] to generate CBI Deleted the [ ] codon in place of the original [ ]. It also contains a second CBI Deleted nucleotide change, silent at the amino acid level, to modify a base of the sequence

recognized by the gRNA; so once the editing is done, the nuclease would stop cutting at that site. Both changes are in frame with the reading frame of the gene.

6. RNP preparation. The RNP used in the bombardment is composed of:
    a. Cas9 nuclease. The process for obtaining the nuclease includes a treatment with DNAses to avoid the presence of DNA in the preparations.
    b. The gRNA. It directs the nuclease to the specific cleavage site at position [ ] CBI Deleted (CDS) of the [ ] gene. CBI Deleted
7. Bombardment. The RNPs and the repair template (BH1070) are adsorbed on the gold particles and bombard onto the soybean embryonic axes.
8. Bombarded axes regeneration in a selective medium. The trait to be introduced through [ ] gene edition is resistance to PPO-inhibiting herbicides. The CBI Deleted herbicide [ ] is used as a selective agent during the regeneration process. CBI Deleted
9. T0 genotyping by Sanger sequencing of the [ ] gene and selection of CBI Deleted the edited events.
10. T1 seed harvest from edited events carrying the mutation [ ]. CBI Deleted
11. Growth and development of edited T1 plants.
12. Tolerance test in T1 plants to various PPO inhibitors of the [ ] and CBI Deleted [ ] families. CBI Deleted
13. Sequencing of the [ ] gene in T1 plants that showed tolerance to the CBI Deleted herbicides tested.

c. Description of the intended genetic modification

A DNA DSB will be made within the *ppo2* gene using Cas9 nuclease and will be repaired through a HDR pathway in the presence of the supplied DNA repair template. The ssDNA consisted of the exact same sequence as the targeted sequence near the DSB except for the specific base pair substitutions and flanked on each side by homology arms (sequences identical to the sequences immediately upstream and downstream of the genomic sequence to be edited) (Figure 1). The [ ] substitution is accomplished by changing the third base [ ] in CBI Deleted the [ ] coding triplet to [ ] in the [ ] coding triplet. Additionally, CBI Deleted the change of nucleotide [ ] is expected at position [ ] of the (CDS) *ppo2* gene, which CBI Deleted does not generate changes at the amino acid level, conserving the amino acid [ ]. CBI Deleted

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Figure 1. Sequence rendering shows the partial sequence of soybean genome sequence, repair DNA (BH1070) and gRNA (gRNA72) directing Cas9 nuclease to make the double stranded break (DSB).

d. Description of the screening tests to confirm edition in the the targeted gene

Specific primers will be designed to amplify the targeted gene from T0 events and the amplicons obtained will be sequenced by Sanger. The chromatograms will be analyzed and the T0 plants that present a homozygous or heterozygous peak in the target base will be selected and harvested. T1 seeds will be tested for herbicide tolerance to various PPO inhibitors; herbicide resistant T1 plants will be genotyped through Sanger to select the positive ones that carry the [ ] mutation in homozygous. CBI Deleted

There is one additional protoporphyrinogen oxidase gene paralog in the soybean genome, identified as *ppo1* [ ]. Partial sequence alignment of these two genes was performed and showed no significant similarity. gRNA72 does not have sequence complementarity with this paralog gene, therefore it does not constitute a possible off-target of the system. In addition, a genome blast was performed and no potential off-targets for gRNA72 were found in the soybean genome. CBI Deleted

D. Description of the trait

The trait introduced by gene editing is herbicide resistance. Naturally-occur mutations showed that specific amino acids substitutions within PPO protein can confer herbicide tolerance to PPO inhibitors[12–14] in different crops. The gene targeted for genome editing is *ppo* gene [ ], which encodes for protoporphyrinogen oxidase (PPO; EC 1.3.3.4) that oxidates the protoporphyrinogen to protoporphyrin IX. Protoporphyrin IX is an important precursor to biologically essential prosthetic groups such as heme, cytochrome C, and chlorophylls. In plants, the interruption of porphyrin metabolism is lethal and the PPO enzyme is the target of herbicides classified as PPO inhibitors, such as diphenyl ethers, oxadiazoles, phenyl phthalimide and triazolinone, which act as competitive inhibitors with the enzyme CBI Deleted

substrate. For example, the R128G mutation in *A. retroflexus* confers resistance to PPO inhibitors from the group of diphenyl ethers [14].

E. References

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Thank you for your time in reviewing this request and I look forward to your response.

Sincerely Yours,

Dr. Carlos Perez

President, Bioheuris Inc.
1100 Corporate Square Dr., Rm 236.
St. Louis, MO 63132
Cell: 54 9 341 3831720
Email: carlos.perez@bioheuris.com

Section 1: Sequence

1. **Nucleotide sequence corresponding to *Glycine max ppo2* gene.**

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[ ]: nucleotide to be modified by [ ] after edition with the BH1070 repair template CBI Deleted

[ ]: nucleotide to be modified by [ ] after edition with the BH1070 repair template BH1070. CBI Deleted

atg: Transcription start codon.

tga: transcription termination codon.

2. Amino acid sequence corresponding to *Glycine max* PPO2.

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[ ]: [ ] that will be substituted for [ ] as a result of gene editing. CBI Deleted

3. gRNA guide sequence:

>gRNA72
[ ] CBI Deleted

4. Real template sequence BH1070:

>BH1070
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