January 23, 2024

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737
Re: CBI Justification

Dear Bernardette

In this Regulatory Status Review no. **23-195-01rsr** we have reserved information regarding: the mutations to be introduced, the gene to be edited and the final sequence to obtain.

Our justification for keeping this information confidential is that BioHeuris treats the information it has labeled as CBI both customarily and actually as private, and this information has been provided to the U.S. government under an assurance of privacy. The information is "confidential" within the meaning of 5 U.S.Code §552(b)(4), the Freedom of Information Act's Exemption 4.

We hope you can understand our justification and are available for any further discussion.

Thanks in advance

DocuSigned by:
M. Celeste Varela
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Dr. Celeste Varela

July 13, 2023
Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737
Re: Request for Confirmation of Exemption

RECEIVED
By llightle at 1:04 pm, Jul 14, 2023

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 are developing using Base Editing CRISPR-Cas9-mediated gene editing technology. The proposed soybean product contains two targeted base pair substitution (edits) in the 4-hydroxyphenylpyruvate dioxygenase (HPPD) gene gene and is tolerant to herbicides that otherwise kill plants through inhibition of the HPPD 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 substitution in the same codon (edits) in the 4-hydroxyphenylpyruvate dioxygenase (HPPD) 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.

D. 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

BioHeuris have been developing soybean (*Glycine max*) plants using Base Editing technology. This technology is based on the use of a catalytically impaired Cas9 (nCas9) nuclease that is unable to create DNA double-strand breaks, fused to a cytidine deaminase (APOBEC) (Komor *et al.*, 2016; Zhu *et al.*, 2020). Soybean tissues will be transformed with the corresponding DNA vector (described below) using Agrobacterium-mediated transformation followed by plant regeneration. BioHeuri's HPPD herbicide tolerant soybean will contain the point mutation/substitution [AAG] instead of the wild-type codon [ ]. This mutation changes the [CBI Deleted]. The final product, obtained by this strategy will not CBI Deleted

contain any foreign DNA in the soybean genome therefore will not constitute a new genetic combination.

Steps involved to obtain the intended soybean plant:

1. Vector construction with ABE machinery (Fig. 1). gRNA design to target [ ] in the soybean *hppd* gene [ ]. CBI Deleted CBI Deleted

   Vector features and coordinates:

   - pVS1 StaA (stability protein from the Pseudomonas plasmid pVS1) (1656…2285)
   - pVS1 RepA (replication protein from the Pseudomonas plasmid pVS1) (2714…3787)
   - pVS1 (OriV, origen de replicación del plásmido pVS1 de Pseudomonas) (3853…4047)
   - bom (basis of mobility region from pBR322) (4391…4531)
   - Ori (high-copy-number ColE1/pMB1/pBR322/pUC origin of replication) (4717…5305)
   - KanR (aminoglycoside phosphotransferase) (5392…6186)
   - RB T-DNA repeat (right border repeat from nopaline C58 T-DNA) (332…356)
   - LB T-DNA repeat (left border repeat from nopaline C58 T-DNA) (6611…6635)
   - CaMV35S enhanced (cauliflower mosaic virus 35S promoter with a duplicated enhancer region) (7786…8463)
   - Spec R (6927…7718)
   - CaMV poly(A) signal (cauliflower mosaic virus polyadenylation signal) (6713…6887)
   - AtU6 (U6 promoter from Arabidopsis thaliana) (8574…8998)
   - gRNA261 (8999…9018)
   - gRNA_Scaffold (9019…9094)
   - U6-26t plus (9095…9286)
   - GmUbiXL Pro+5U (soybean UbiXL gene promoter) (9303…11729)
   - APOBEC-1 (cytidine deaminase (C to U editing enzyme) from rat) (11745…12.431)
   - nCas9 (nickase mutant (D10A) of the Cas9 endonuclease from the Streptococcus pyogenes Type II CRISPR/Cas system) (12480…16.580)
   - UGI (uracil-DNA glycosylase inhibitor from a Bacillus subtilis bacteriophage) (16593. 16.841)
   - SV40NLS (nuclear localization signal of SV40 (simian virus 40) large T antigen) (16854…16.874)
   - CaMV35S 3U+Ter

2. Soybean embryonic axes from mature seed obtention.

3. Transformation of soybean embryonic axes mediated by *Agrobacterium tumefaciens* (EHA105) carrying the base editing vector pBH584.
4. Spectinomycin selection in the transformed explants and transgenic plant regeneration (T0).
5. T0 genotyping by Sanger sequencing of the *Gmhppd* gene and selection of the edited events.
6. T1 seed harvest from edited events carrying the mutation [ ]. CBI Deleted
7. T1 genotyping by Sanger sequencing of the *Gmhppd* gene and selection of the edited events.
8. T1 genotyping to sort-out the transgenic plants, using 10 sets of primers to detect specific features of the CRISPR-Cas construct. According to the Mendelian segregation laws, 25% of the plants should not contain the transgene in the T1 generation.
9. Growth and development of T1 transgene-free edited plants and seeds harvest.
10. Herbicide tolerance assay to HPPD inhibitors family in T2 plants.

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Figure 1. Vector map harboring the base editing machinery, the gRNA and the resistance cassette (full sequence in Section 1).

c. Description of the intended genetic modification

Two base substitution ([ ], [ ] on the complementary strand) will be made within the *hppd* gene using a catalytically impair Cas9 nuclease (nCas9) fused to a cytidine deaminase enzyme that retain the ability to be programmed with a gRNA and do not induce dsDNA breaks (Komor *et al.*, 2016). The resulting "base editor" (CBE) mediates the direct conversion of cytidine to uridine, thereby effecting a C→T (or G→A) substitution, thus converting cytidines within a window of approximately seven nucleotides. CBI Deleted

The [ ] substitution is accomplished by changing the first two bases [ ] in the [ ] coding triplet to [ ] in the [ ] coding triplet. CBI Deleted CBI Deleted

The potential off-target sites for gRNA261 were analyzed using CRISPR RGEN tool software (Table 1). There was one potential off-target with zero mismatches consisting of the [ ] paralog gene: [ ]. CBI Deleted

Target sites and potential off-target sites	Site	Position	Target and off-target sequences	Num of mismatches
[ ]	Target	2204671	AACCCCCACATGCACCCTTC	0
[ ]	Off-target	46604984	AACCCCCACATGCACCCTTC	0

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Table 1. Potential off-target sites of gRNA261 (mismatches marked in red)

Both genes share high DNA sequence homology, a partial alignment of the target region is shown in Figure 2. To guarantee that [ ] has not been edited in our final product, the target edited plants will be subjected to Sanger sequencing at the corresponding position with specific primers (Table 2). Edited plants in the off-target site will be discarded. CBI Deleted

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Figure 2: Partial DNA sequence alignment of: [CBI Deleted]. The gRNA261 hybridization region is CBI Deleted shown in orange.

Primer Name	Sequence	Tm	Product size
OT1_Fw	GCCTGTTTACGGAACGAAAC	57°C	562 bp
OT2_Rv	TGAAAATACTGCTGCACTGTCATAG	58 °C	

Table 2: Primer sets to identify off-target mutation.

d. Description of the screening tests to confirm the targeted gene editing of *hppd* gene

Specific primers will be designed to amplify the *hppd* gene from T0 events and the amplicons obtained will be sequenced by Sanger. The chromatograms will be analyzed and the events that present a homozygous or heterozygous peak in the target base will be selected and harvested. T1 plants will be genotyped through Sanger to select the positive ones that carry the [ ] mutation. CBI Deleted

To confirm there is no remaining exogenous vector DNA in edited soybean plants, ten specific primer pairs will be used to amplify different components of the CRISPR-Cas construct (Table 3) in the progeny (T1 plants). The final product will not contain any foreign DNA; only edited plants with no detectable PCR amplification will be maintained. Additionally, a WGS approach could be implemented, if required.

Transgene-free edited plants will be selected to drive to T2 harvest. Finally, an herbicide tolerance assay to HPPD inhibitors family in T2 plants will be performed.

Primer Name	Sequence	Tm	Product size
AtU6_Fw	GACTTGCCTTCCGCACAATACATCATTTC	60°C	506 bp
scaffold_Rv	CCACTTTTTCAAGTTGATAACGGACTAGC	58°C	
GmUBI3_Fw	CTCCTCCTTCCTTCGCAGTTCAATTC	59.3°C	578 bp
GmUBI3_Rv	ACCCTAATCCACGGCAATAGAATCAAC	58.7°C	
CBE_Fw	TTGGTTCCTTAGCTGGTCACCGT	60,5ºC	578 bp
nCas9.1_Rv	CCTTGAACTTCTTAGATGGCACCTTG	57,7ºC	
nCas9.2_Fw	AAGAACGGATACGCTGGTTACAT	59°C	509 bp
nCas9.2_Rv	GTCAACTCGTTGTACACGGTGAAGT	61°C	
nCas9.3_Fw	CTCGCTGGATCTCCTGCAATCA	59.1°C	499 bp
nCas9.3_Rv	TGATGAGCTTAGCGTTGAGGAGC	59.1°C	

SV40_Fw	CCTAAGAAGAAGAGAAAGGTGTAGTGT	59ºC	489 bp
tNOS_Rv	TGATAATCATCGCAAGACCGGCAAC	62ºC	
RBtDNA_Fw	TGACAGGATATATTGGCGGGTAAACC	58,4ºC	595 bp
backbone_Rv	GCTCGGCAATGTCCAGTAGG	58,2ºC	
pVS1RepA_Fw	GCCGATGGCGGAAAGCAGAAAG	61.3 °C	655 bp
pVS1oriV_Rv	ACGGGTTCCGGTTCCCAATGTA	60.8 °C	
KanR_Fw	TACCTTAGCAGGAGACATTCCTTCCG	59,6ºC	542 bp
LBtDNA_Rv	GTTTACACCACAATATATCCTGCCACC	57,6ºC	
SpecR_Fw	ACATCCTTCGGCGCGATTTTG	58,6 °C	499 bp
SpecR_Rv	GCCTGAAGCCACACAGTGATATTG	58.2 °C	

Table 3: Primer sets to confirm the absence of transgenic lines.

E. Description of the new trait

The trait introduced by gene editing is herbicide resistance. Mutations generated by particular base transitions into the HPPD protein of *O. sativa* and *T. aestivum* by using base editors was demonstrated (Zong *et al*., 2018). Also, mutated HPPD enzymes have been obtained with reduced sensitivity to HPPD-inhibiting herbicides. For example, an HPPD mutant G336W derived from *Pseudomonas fluorescens* strain A32 was obtained (Matringe *et al*., 2005). This single amino acid change in the active site led to reduced sensitivity to isoxafutole and a moderate impact on HPPD enzymatic activity. Another example is an HPPD isoenzyme (called AvHPPD-03) obtained from oats (*Avena sativa*), which showed tolerance to mesotrione (Kramer *et al*., 2014; Siehl *et al*., 2014). This isoenzyme has a single amino acid deletion in the N-terminal domain (A111). Particularly, both genes (PfHPPD W336 and AvHPPD-03) have been successfully used for the development of genetically modified crops, such as soybean and cotton (Dreesen *et al*., 2018).

The gene targeted for genome editing is *Gmhppd* [.], which encodes CBI Deleted for 4-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) that catalyzes the second step in the tyrosine catabolism pathway, converts 4-hydroxyphenylpyruvate (HPP) to homogentisate, which is a precursor for plastoquinone and tocopherol biosynthesis. HPPD is the target site of herbicides from different chemical families such as isoxazoles (isoxaflutole and pyrasulfotole), pyrazolones (topramezone) and triketones (mesotrione, bicyclopyrone and tembotrione) (van Almsick, 2009). After the treatment with these herbicides, susceptible plants exhibit bleaching symptoms as a result of loss of carotenoid synthesis and eventually lead to lipid peroxidation of cell membranes.

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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

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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 hppd* gene.

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[ ]: nucleotides to be modified by [ ] after base editing ([ ] on the complementary CBI Deleted strand).

atg: Transcription start codon.

tga: transcription termination codon.

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

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

3. **Full vector sequence**
   >pBH584
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Sets of primers that will be used to detect transgenic events

AtU6_Fw:	GACTTGCCTTCCGCACAATACATCATTTC
scaffoldGm_Rv:	CCACTTTTTCAAGTTGATAACGGACTAGC
GmUBI3_Fw:	CTCCTCCTTCCTTCGCAGTTCAATTC
GmUBI3_Rv:	ACCCTAATCCACGGCAATAGAATCAAC
CBE_Fw:	TTGGTTCCTTAGCTGGTCACCGT
nCas9.1_Rv:	CCTTGAACTTCTTAGATGGCACCTTG
nCas9.2_Fw:	AAGAACGGATACGCTGGTTACAT
nCas9.2_Rv:	GTCAACTCGTTGTACACGGTGAAGT
nCas9.3_Fw:	CTCGCTGGATCTCCTGCAATCA
nCas9.3_Rv:	TGATGAGCTTAGCGTTGAGGAGC
SV40_Fw:	CCTAAGAAGAAGAGAAAGGTGTAGTGT
tNOS_Rv:	TGATAATCATCGCAAGACCGGCAAC
RBtDNA_Fw:	TGACAGGATATATTGGCGGGTAAACC
backbone_Rv:	GCTCGGCAATGTCCAGTAGG
pVS1RepA_Fw:	GCCGATGGCGGAAAGCAGAAAG
pVS1oriV_Rv:	ACGGGTTCCGGTTCCCAATGTA
KanR_Fw:	TACCTTAGCAGGAGACATTCCTTCCG
LBtDNA_Rv:	GTTTACACCACAATATATCCTGCCACC
SpecR_Fw:	ACATCCTTCGGCGCGATTTTG
SpecR_Rv:	GCCTGAAGCCACACAGTGATATTG