November 25, 2022
Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737
Re: Request for Confirmation of Exemption

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By ajdrummond for BRS Document Control Officer at 4:26 pm, Nov 25, 2022

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 Base Editing CRISPR-Cas9-mediated gene editing technology. The proposed soybean product contains one 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. The request is described as follows.

A. Requestor's name and contact information

Dr. Carlos Perez
Bioheuris Inc.
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Email: carlos.perez@bioheuris.com

B. Description of plant's genus, species

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

C. Regulatory exemption we are claiming

As described below, our intended final plant line has one amino acid substitution resulting from only one base pair change made to the soybean genome. We think that our product concept is eligible for exemption from regulation under the new BRS SECURE rule published in 7 CFR part 340 section, more specifically §340.1(b)2, which states "the genetic modification is a targeted single base pair substitution" could be exempted from regulation.

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 [2–4] in different crops. The gene targeted for genome editing is *ppo2* [ ], which encodes for protoporphyrinogen oxidase (PPO; EC CBI Deleted 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 substrate[5]. For example, the R128G mutation in *A. retroflexus* confers resistance to PPO inhibitors from the group of diphenyl ethers[6].

E. Description of reagents used for genome editing

BioHeuris intends to develop 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 an adenosine deaminase (ecTadA heterodimer-7.10)[1,7] which introduces precise changes at the target nucleotide base. The recently developed adenine base editors (ABEs), which convert an adenosine (A) nucleotide to a guanosine (G), offer an alternative to generate rapidly dominant traits, including herbicide-tolerant traits[8]. Particularly, it is possible to introduce specific mutations in *G. max* genes to obtain the desired trait by using base-editing editors [9].

Soybean tissues will be transformed with the corresponding DNA vector (described below) using Agrobacterium-mediated transformation followed by plant regeneration. BioHeuris' PPO herbicide tolerant soybean will contain the point mutation/substitution [ ] CBI Deleted instead of the wild-type codon [ ]. This mutation changes the [CBI Deleted]. The final product, obtained by this strategy will not contain any foreign CBI Deleted 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 position [ ] in the soybean *ppo2* gene [ ]. 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)
- gRNA260 (8999…9018)
- gRNA_Scaffold (9019…9094)
- GmUbi3 (soybean Ubiquitin-3 gene promoter) (9527…10443)
- ABE 7.10 (ecTadA heterodimer-7.10, A to G editing enzyme) (10466…11656)
- nCas9-VQR (nickase mutant (D10A) of the Cas9 endonuclease from the *Streptococcus pyogenes* Type II CRISPR/Cas system with the VQR mutation) (11657…15757)
- SV40NLS (nuclear localization signal of SV40 (simian virus 40) large T antigen) (15770…15790)
- CaMV poly(A) signal (cauliflower mosaic virus polyadenylation signal) (15860…16036)

2. Soybean embryonic axes from mature seed obtention.
3. Transformation of soybean embryonic axes mediated by *Agrobacterium tumefaciens* (EHA105) carrying the base editing vector (pBH583).
4. Spectinomycin selection in the transformed explants and transgenic plant regeneration (T0).
5. T0 genotyping by Sanger sequencing of the *ppo2* 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 *ppo2* 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 PPO inhibitors family in T2 plants.

F. Description of the intended genetic modification

A precise base substitution ([ ] to [ ]) will be made within the *ppo2* gene using a CBI Deleted catalytically impair Cas9 nuclease with the VQR mutation (nCas9-VQR) fused to an adenosine deaminase enzyme that retain the ability to be programmed with a gRNA and do not induce dsDNA breaks[1]. The resulting “base editor” (ABE) mediates the direct conversion of adenosines to guanosines, thereby effecting a A→G (or T→C) substitution, thus converting adenosines within a window of approximately eight nucleotides[10].

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

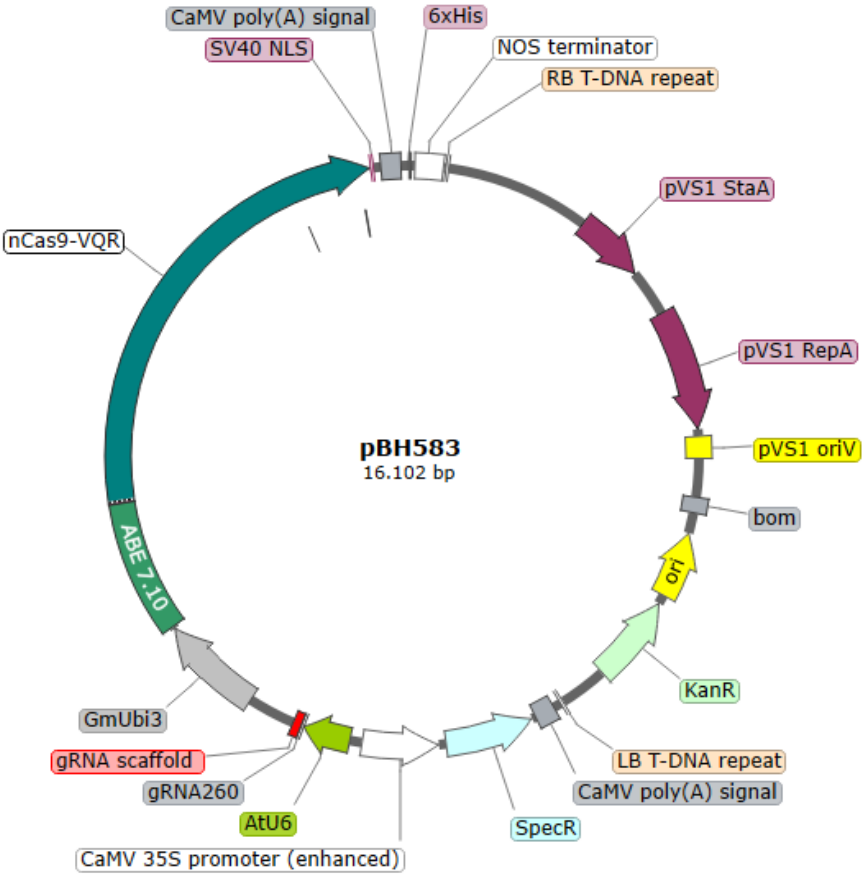

Figure 1. Vector map harboring the base editing machinery, the gRNA and the resistance cassette (full sequence in Section 1).

G. Description of the screening tests to confirm the targeted gene editing of *ppo2* gene

There are two protoporphyrinogen oxidase genes paralog [CBI Deleted] in the soybean genome which shares CBI Deleted very low DNA sequence homology with *ppo2*. The nucleotide alignment between *ppo2* and its paralogs leaves non-significant alignment. An amino acid alignment of the three proteins is represented in Figure 2 to show the low homology between them. The target amino acid for base editing is shown in red.

[

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]

Figure 2. Partial amino acid sequence alignment of: [

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]

Furthermore, additional off-targets were searched with Cas-OFFinder[11] software. A unique potential off target with 2 mismatches was found, located on chromosome [, CBI Deleted] which corresponds to an intron of the gene [ ]. To CBI Deleted guarantee that [ ] has not been edited in our final product, the edited plants CBI Deleted will be subjected to Sanger sequencing at the corresponding position with specific primers (Table 1; Figure 3). Edited plants will be discarded.

Primer Name	Sequence	Tm	Product size
OT1_Fw	AAGACCACATGTATCCTCCATCCT	59°C	507 bp
OF1_Rv	GGTGAAGCAGTTTGTGGAGGTT	60°C	

Table 1: Primer sets to identify off-target mutation.

[

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]

Figure 3: Potential off target sequence for gRNA260.

Specific primers will be designed to amplify the *ppo2* target 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 2) 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 PPO 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	
ABE_Fw	AAACGTGCGTGGGACGAGAGAG	61,5ºC	911 bp
ABE_Rv	CCAGGGTAGTGGAGAACGTCCATAAGA	60,7ºC	
nCasVQR.1_Fw	ATCTTCAGCAACGAGATGGCCAAG	59.5ºC	524bp
nCasVQR.1_Rv	GCCAGGTCGAAGTTGCTCTTGAA	59,7ºC	
nCasVQR.2_Fw	GCTTCGCCAACCGCAACTTCAT	61°C	593 bp
nCasVQR.2_Rv	GCGCCAGTAGTTCTTCATCTTCTTCAC	69.3°C	
SV40_Fw	CGACTCTGGTGGTTCTCCAAAGAAA	59ºC	476 bp
tNOS_Rv	TGATAATCATCGCAAGACCGGCAAC	59,5ºC	
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 2: Primer sets to confirm the absence of transgenic lines.

H. References

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11. Bae, S., Park, J. & Kim, J.-S. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinforma. Oxf. Engl.* **30**, 1473–1475 (2014).

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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Section 1: Sequence

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

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

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 base editing. CBI Deleted

3. **Full vector sequence**

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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
ABE_Fw:	AAACGTGCGTGGGACGAGAGAG
ABE_Rv:	CCAGGGTAGTGGAGAACGTCCATAAGA
nCasVQR.1_Fw:	ATCTTCAGCAACGAGATGGCCAAG
nCasVQR.1_Rv:	GCCAGGTCGAAGTTGCTCTTGAA
nCasVQR.2_Fw:	GCTTCGCCAACCGCAACTTCAT
nCasVQR.2_Rv:	GCGCCAGTAGTTCTTCATCTTCTTCAC
SV40_Fw:	CGACTCTGGTGGTTCTCCAAAGAAA
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