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

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 rice (*Oryza sativa*) we intend to develop using Base Editing CRISPR-Cas9-mediated gene editing technology. The proposed rice product contains one targeted base pair substitution (edits) in the Acetolactate Synthase (*ALS*) gene[1] and is tolerant to herbicides that otherwise kill plants through inhibition of the essential ALS 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:*Liliopsida*
- Family: *Poaceae*
- Genus: *Oryza*
- Species: *Oryza sativa; L*
- Subespecies*: indica*
-

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 rice 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 ALS protein can confer herbicide tolerance to ALS inhibitors (Powels *et al.*, 2010) in different crops. The gene targeted for genome editing is *als* [ ], which encodes for Acetolactate Synthase that catalyzes the first CBI Deleted step in the pathway of biosynthesis of branched-chain essential amino acids valine, leucine and isoleucine, and is present in the chloroplasts of all plant species. Herbicides from the five chemical groups sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidinyl-thiobenzoates and sulfonyl-aminocarbonyl-trizolinone inhibit ALS and cause plant death by deprivation of branched chain amino acids.

E. Description of reagents used for genome editing

BioHeuris intends to develop rice (*Oryza sativa*) 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) which introduces precise changes at the target nucleotide base. The recently developed cytidine base editors (CBEs), which convert a cytosine (C) nucleotide to a thymine (T), offer an alternative to generate rapidly dominant traits, including herbicide-tolerant traits (Mishra *et al.* 2020). CBEs have been used to generate herbicide-tolerant rice by introducing particular base transitions in *als* gene while the ALS protein retained its activity (Shimatani *et al.*, 2017) (Kuang *et al.*, 2020) (Zhang *et al.*, 2017) (Zhang *et al.*, 2020). Rice tissues will be transformed with the corresponding DNA vector (described below) using *Agrobacterium*-mediated transformation followed by plant regeneration. BioHeuris' ALS herbicide tolerant rice will contain the point mutation/substitution [ ] instead CBI Deleted of the wild-type codon [ ]. This mutation changes the [ ]. CBI Deleted The final product, obtained by this strategy will not contain any foreign DNA in the rice genome therefore will not constitute a new genetic combination.

Steps involved to obtain the intended rice plant:

1. Vector construction with CBE machinery (Fig. 1). gRNA design to target [ ] in the CBI Deleted rice *als1* gene [ ]. CBI Deleted

   Vector features and coordinates

   - pVS1 StaA (stability protein from the Pseudomonas plasmid pVS1) (1233…1862)
   - pVS1 RepA (replication protein from the Pseudomonas plasmid pVS1) (2291…3364)
   - pVS1 (OriV, replication origen from the Pseudomonas plasmid pVS1) (3430…3624)
   - bom (basis of mobility region from pBR322) (3968…4108)
   - Ori (high-copy-number ColE1/pMB1/pBR322/pUC origin of replication) (4294…4882)
   - KanR (aminoglycoside phosphotransferase) (4969…5763)

- RB T-DNA repeat (right border repeat from nopaline C58 T-DNA) (22786…22810)
- LB T-DNA repeat (left border repeat from nopaline C58 T-DNA) (6188…6212)
- PvUbi2 (Panicum virgatum Ubi2 promoter) (7537…9397)
- HygR (6505…7530)
- CaMV poly(A) signal (cauliflower mosaic virus polyadenylation signal) (6290…6464)
- ZmUbi promoter (9623…11614)
- mutGRF4-GIF1_3xHA (11656...13710)
- NOS terminator (nopaline synthase terminator and poly(A) signal) (13744…13996)
- OsU3 Oryza sativa (rice) snRNA U3 promoter (14095…14474)
- gRNA_Scaffold (14476…14581)
- Maize polyubiquitin gene promoter (14883…16875)
- SV40 NLS_APOBEC-1 (cytidine deaminase (C to U editing enzyme) from rat)_XTEN (16886…17641)
- Cas9(D10A) (nickase mutant of the Cas9 endonuclease from the Streptococcus pyogenes Type II CRISPR/Cas system) (17642…21742)
- Nucleoplasmin NLS_UGI_SV40 NLS (21743…22096)
- E9 terminator (22104…22736)

2. Explant (scutellar embryogenic calli) preparation from rice mature seeds.
3. *Agrobacterium*-mediated transformation with the pBH501.
4. Hygromycin selection of the transformed calli and regeneration of T0 transgenic plants.
5. T0 genotyping by PCR and restriction enzymes followed by Sanger sequencing of the *als* gene [(LOC_Os02g30630)] to confirm edited plants.
6. Backcrossing to obtain T1 plants [ ] transgenic free (25%). CBI Deleted
7. T1 genotyping to sort-out the transgenic plants, using 3 sets of primers to detect specific features of the vector (e.g. Hygromycin, nCas9, CBE).
8. Growth and development of T1 transgene-free edited plants and seeds harvest.
9. Herbicide tolerance assay to ALS inhibitors family in T2 plants.

F. Description of the intended genetic modification

A precise base substitution [ ], will be made within the *als* gene using a CBI Deleted 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 five nucleotides. The [ ] substitution is accomplished by changing [CBI Deleted]. CBI Deleted

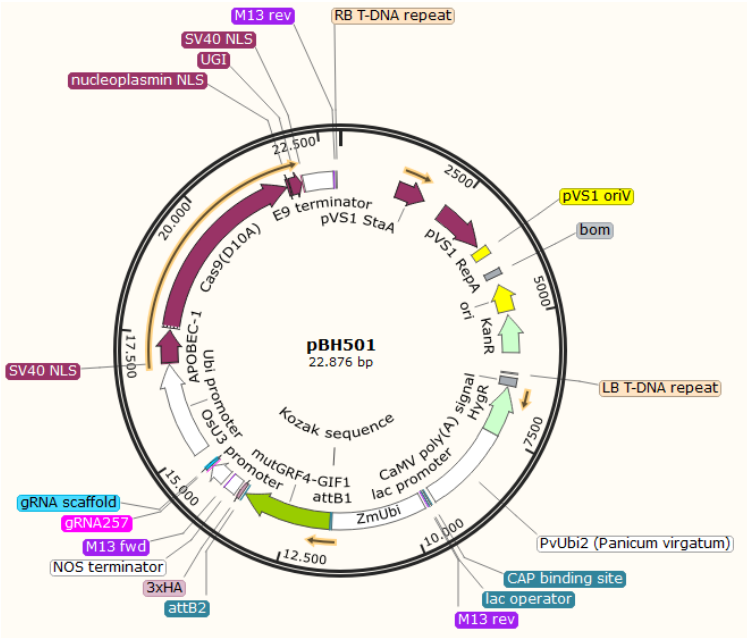

Figure 1. Vector map harboring the base editing machinery, the gRNA and the resistance cassette (full sequence in Section 1).

G. Description of the screening tests to confirm the targeted gene editing of *als1* gene

In rice, there are three ALS homologs: *als1* [ ], *als2* [ ], and *als3* [ ]. A partial sequence alignment of these three alleles are represented in Figure 2. To ensure that the final edited product is only present in the *als1* target gene, this allele will be amplified with sequence-specific primers for direct Sanger sequencing (P1 and P2 indicated in Figure 2) and if necessary it would be combined with locked nucleic acid (LNA) probes (IDT). Additionally, off-targets sites containing one to four nucleotide mismatches will be examined by Sanger sequencing. The primer sets to be used are listed in Table 1.

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Target site and potential off-target site	Locus	Target and off-target sequence	No. of mismatches
OsALS1-P171 (*als1*)		CAGGTCCCCCGCCGCATGATCGG	
P171-OFF1 (*als3*)	4:-19143497	CAGGTCCCGCGCCGCATGATCGG	1
P171-OFF2 (*als2*)	4:+19169754	CAGGCCCCGCGCCGCATGATCGG	2
P171-OFF3	5:+27073157	CCGGTTCGCCGCCTCATGATTGG	4
P171-OFF4	8:+1628685	CAGATCCGCCGCCGCACGCTCGG	4
P171-OFF5	5:-19659371	CAGTTCCTCCGCAGCATCATCGG	4

Table 1: Potential off-target sites for the ALS1-P171.

[

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Figure 2. Partial sequence alignment of *als1* target gene [ ] and its two homologs (*als2* and *als3*). ALS homologs are highly conserved at the region of interest; the gRNA was specifically designed for nuclease/gRNA complex cuts the *als1* gene, however, it could target the *als2* and *als3* but with less efficiency compared to *als1.* P1, P2: primers for specific amplification of the *als1* gene (Target). CBI Deleted

Conventional breeding will be used to generate null-segregant progeny that contains the intended mutation without introduced exogenous DNA. To confirm there is no remaining exogenous vector DNA in edited rice plants, thirteen 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, if required, a WGS approach could be designed and implemented.

Primer Name	Sequence	Tm	Product size
CaMV_polyA_Fw	CAACACATGAGCGAAACCCT	66 °C	683 bp
Hygro_Rv	CGATTGCTGATCCCCATGTG	66 °C	
Hygro_Fw	CAGGACATATCCACGCCCTC	60 °C	904 bp
PvUbi2_Rv	CTGCCCTGTTTTTGGGTTCG	60 °C	
PvUbi2_Fw	GGGAAGAGTTTTGAGCAGCC	66 ºC	601 bp
lac_promoter_Rv	CACAACATACGAGCCGGAAG	66 ºC	
ZmUbi_Fw	CGATGCTCACCCTGTTGTTT	66 ºC	650 bp

GRF4-GIF1_Rv	AGAGGGAGTGGTTGTGGAAG	67 ºC	
GRF4-GIF1_Fw	GCTACGCTTCCTCTACCACT	66 ºC	632 bp
GRF4-GIF1_3'_Rv	CTAGCTTCCTTCCTCCTCGG	67 ºC	
OsU3_Fw	CAGGCATGCATGGATCTTGG	67 ºC	629 bp
backbone_Rv	CCAGTGTTGTGTGAAGCCAA	66 ºC	
ZmUbi_Fw	CGATGCTCACCCTGTTGTTT	66 ºC	652 bp
APOBEC-1_Rv	CAGGATGTTGAGGCATGGTG	66 ºC	
nCas9_Fw	TTCAGAATCCCATACTACGTCGGCCC	60 ºC	1031 bp
nCas9_Rv	TTGATGCCCTCCTCGATGCGCTTCA	60 ºC	
UGI_Fw	ACCAACCTGTCCGACATCAT	66 ºC	640 bp
E9_terminator_Rv	GAGGCCACGATTTGACACAT	65 ºC	
E9_terminator_Fw	TCAGACCTAGAAAAGCTGCAA	63 ºC	276 bp
RB_T-DNA_Rv	ACCCGCCAATATATCCTGTCA	66 ºC	
pVS1RepA_Fw	GCCGATGGCGGAAAGCAGAAAG	63 °C	795 bp
pVS1oriV_Rv	TTATGCACAGGCCAGGCGGG	65 °C	
KanR_Fw	TACCTTAGCAGGAGACATTCCTTCCG	59,6ºC	542 bp
LBtDNA_Rv	GTTTACACCACAATATATCCTGCCACC	57,6ºC	
CaMVpolyA_Fw	TGATAGAAGTATTTTACAAATACAA	50 °C	483 bp
NeoR/KanR_Rv	CCGTGGTTGGCTTGTAT	54 °C	

Table 2: Primer sets to confirm the absence of transgenic lines.

H. References

Kuang, Y., Li, S., Ren, B., Yan, F., Spetz, C., Li, X., Zhou, X., Zhou, H., 2020. Base-Editing-Mediated Artificial Evolution of OsALS1 In Planta to Develop Novel Herbicide-Tolerant Rice Germplasms. Mol. Plant 13, 565–572. https://doi.org/10.1016/j.molp.2020.01.010

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Zhang, Y., Xu, Y., Wang, S., Li, X., Zheng, M., 2017. Resistance mutations of Pro197, Asp376 and Trp574 in the acetohydroxyacid synthase (AHAS) affect pigments, growths, and competitiveness of Descurainia sophia L. Sci. Rep. 7, 16380. https://doi.org/10.1038/s41598-017-16655-0

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

Sincerely Yours,

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Section 1: Sequence

1. **Genomic sequence corresponding to Oryza sativa als1 (*Osals1)* gene.**

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[ ]: [ ] to be modified by CBE and converted to [ ]. CBI Deleted

2. **Nucleotide coding sequence corresponding to Oryza sativa als1 (*Osals1)* gene.**

>OsALS1 [ ](CDS) CBI Deleted

[

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]

[ ]: [ ] to be modified by CBE and converted to [ ]. CBI Deleted

ATG: Start codon

TAA: Stop codon

3. **Amino acid sequence corresponding to *Oryza sativa* ALS1.**

>[ ] CBI Deleted

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[ ]: [ ] that will be modified to [ ] after base editing CBI Deleted

4. **Full vector sequence**

>pBH501

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