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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

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Dear Ms. Juarez,

Bioheuris Inc. respectfully requests a petition for determination on the regulatory status of a soybean (*Glycine max*) that we intend to develop using Non-Homologous End-Joining (NHEJ), CRISPR-Cas9-mediated gene editing technology. The proposed soybean product contains an in-frame deletion (genetic modification) in the auxin co-receptor (AUX/IAA), resulting from cellular repair of a double targeted DNA break, in the absence of an externally provided repair template, that confers resistance to auxinic herbicides. 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 has an in-frame deletion mutation made to the soybean genome. 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.
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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

In 2005, a population of *Sisymbrium orientale* weeds from South Africa, also known as Indian hedge mustard, was reported to be resistant to two auxin herbicides (2,4-D and 2-methyl-4-chlorophenoxyacetic acid)[1]. Studies of the resistant population progeny revealed that the resistance was inherited as a single dominant allele[2]. Also, a transcriptome analysis on recombinant inbred lines derived from a cross between 2,4-D-resistant (R) and -susceptible (S) genotypes shows an in-frame 27-bp deletion (9 amino acids) in the degron tail (DT) of the auxin coreceptor Aux/IAA2 of *S. orientale* (SoIAA2)[3]. This deletion reduced binding of natural and synthetic auxins to TIR1 and conferred cross-resistance to 2,4-D and dicamba. BioHeuris has an exclusive patent license from the State University of Colorado (USA) that protects the deletion described above in the SoIAA2 gene. In cotton, this deletion is equivalent to [18] amino acids, which could be specifically deleted using NHEJ, CRISPR-Cas9-mediated gene editing technology and the T3 descendant transgenic-free, in homozygosity for the deletion will be consider the final product.

b. Second Comparator Plant

Centromere-mediated chromosome elimination (CCE) is a novel breeding method used to accelerate breeding in crops[4]. In CCE, the genetically engineered (GE) parent is engineered in such a way that the heritability of the chromosomes of the GE parent is eliminated, and the resulting descendant does not contain any GE material. Associated with this strategy we found an inquiry of the regulatory status of a plant derived from genetically engineered (GE) plants using CCE technique, from the New Zealand Institute for Plant and Food Research (October, 27, 2011) and the response clarify that *APHIS does not consider the crop progeny created via CCE to be regulated articles*. Comparing the final product obtained in the described strategy with the final product obtained in the strategy proposed in this consultation (T3 descendant transgenic-free, modified cotton) we believe that both final products are comparable.

c. Third Comparator Plant

Wheat seeds (M1) were subjected to ethane methyl-sulfonate (EMS). The subsequent generation of seeds (M2) was grown and treated with two ACCAse inhibitor herbicides. Plants that survived to the herbicide treatment were harvested and their seeds (M3) were also grown and treated with the same ACCAse inhibitor herbicides. After plants exhibiting an increased tolerance to the herbicides were evaluated genotypically (DNA sequencing) with primers that amplify A, B and D wheat genomes. Sequence results were compared to previously cloned non-mutant wheat sequences to determine the presence of mutations. When comparing sequences results to non-mutant sequences, non-synonymous mutations were revealed in the ACCase carboxyltransferase domain, all at position 2004 (Ref: *Alopecurus myosuroides* amino acid numbering system). Initially, the researchers identified three accessions with heterozygous resistance-conferring mutations for each of three ACC1

gene homoeologs in hexaploid wheat (6x = 42) on chromosome 2. Not all homoeologs contribute equally to herbicide resistance, with the mutation on the D homoeolog providing the greatest level of resistance, followed by the same mutation in the A and B homoeologs [5]. Later The Wheat Breeding and Genetics Program at Colorado State University introgressed the mutations from the A and D ACC1 homoeologs into an elite genetic background and developed the initial CoAXium® varieties featuring the mutation in both homoeologs[6]. Comparing the final product obtained in the described traditional mutagenesis strategy with the final product obtained in the strategy proposed in this consultation we believe that both final products are comparable.

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 intends to develop soybean (*Glycine max*) using NHEJ (Non Homologous End Joining) technology, where the DNA vector that carries the editing machinery is introduced by transformation with Agrobacterium in the embryonic axis stage of an in vitro soybean culture system, followed by a regeneration protocol that brings the plants to maturity until obtaining seeds. The operating principle of this technology is based on the use of nucleases that can be directed to a specific site in the genome by RNA guides to make a punctual and specific cut in the double-stranded DNA (DSB). When the cell detects the cut, it triggers the repair mechanisms. In eukaryotes, DSB repair occurs mainly by two pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is the primary cellular repair mechanism of the DSB and can occur at any phase of the cell cycle[3,4]. It is important to highlight in relation to this project that when two or more CRISPR/Cas complexes are directed to nearby sites, the deletion of the gene fragment between two DSBs can be promoted.

Soybean tissues will be transformed with the corresponding DNA vector (described below) using Agrobacterium-mediated transformation followed by plant regeneration. The expected genotype in the soybean plant after gene editing is the deletion of [ ] nucleotides between the positions [ ] (CDS) of the Aux/IAA gene [ ], which is equivalent to [ ] amino acids and respects the amino acid identity of the rest of the protein. The final product, obtained by this strategy will not contain any foreign DNA in the soybean genome therefore will not constitute a new genetic combination.

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Steps involved to obtain the intended soybean plant:

1. Design of two specific gRNAs to target the nuclease cutting site in the positions [ ] (CDS) of the Aux/IAA gene [ ] and gene editing vector construction. CBI Deleted CBI Deleted

   Vector features and coordinates:

   - pVS1 StaA (stability protein from the Pseudomonas plasmid pVS1) (12690…13319)
   - pVS1 RepA (replication protein from the Pseudomonas plasmid pVS1) (351…1424)
   - pVS1 OriV (origin of replication from the Pseudomonas plasmid pVS1) (1490…1684)
   - bom (basis of mobility region from pBR322) (2028…2168)
   - Ori (high-copy-number ColE1/pMB1/pBR322/pUC origin of replication) (2354…2942)
   - KanR (aminoglycoside phosphotransferase) (3029…3823)
   - RB T-DNA repeat (right border repeat from nopaline C58 T-DNA) (12386…12410)
   - LB T-DNA repeat (left border repeat from nopaline C58 T-DNA) (4248…4272)
   - CaMV35S enhanced (cauliflower mosaic virus 35S promoter with a duplicated enhancer region) (5423…6100)
   - Spec R (4564…5355)
   - CaMV poly(A) signal (cauliflower mosaic virus polyadenylation signal) (4350…4524)
   - AtU6 (U6 promoter from Arabidopsis thaliana) (6186…6260)
   - gRNA1 (6263…6282)
   - gRNA_Scaffold (6283…6358)
   - AtU6 (U6 promoter from Arabidopsis thaliana) (6409…6483)
   - gRNA2 (6486…6505)
   - gRNA_Scaffold (6506…6581)
   - GmUbi3 (soybean Ubiquitin-3 gene promoter) (6632…7548)
   - Cas9-SpG (variant of Cas9 endonuclease from the Streptococcus pyogenes Type II CRISPR/Cas system with NGN PAM requirement) (7606…11709)
   - SV40NLS (nuclear localization signal of SV40 (simian virus 40) large T antigen) (11722…11742)
   - CaMV poly(A) signal (cauliflower mosaic virus polyadenylation signal) (11812…11988)

2. Soybean embryonic axes from mature seed obtention.
3. Transformation of soybean embryonic axes mediated by *Agrobacterium tumefaciens* (EHA105) carrying the base editing vector (pBH582).
4. Spectinomycin selection in the transformed explants and transgenic plant regeneration (T0).

5. T0 genotyping by Sanger sequencing of the targeted gene to confirm the intended mutation.
6. T1 seed harvest from edited events carrying the [ ]. CBI Deleted
7. 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.
8. Growth and development of T1 transgene-free edited plants and seeds harvest.
9. Herbicide tolerance assay to auxinics inhibitors family in homozygote and heterozygote T2 plants.

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

c. Description of the intended genetic modification

The expected genetic modification in the soybean plant after gene editing is the deletion of [ ] nucleotides between the positions [ ] (CDS) of the Aux/IAA gene, which is equivalent to [ ] amino acids and respects the amino acid identity of the rest of the protein. The gRNA sequences used to modify the [ ] gene have been designed to only make this change in the amino acid sequence of the protein. Additionally, off-target sites containing one or two nucleotide mismatches will be examined by Sanger sequencing at the corresponding position with specific primers. The potential off-target sites of gRNA1 and gRNA2

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found using CRISPR RGEN tool software are listed in Table 1 and Table 2, respectively. Edited plants in off-target sites will be discarded.

Target sites and potential off-target sites	Locus	Target and off-target sequences (5'–>3')	Num of mismatches
[ ]	2:-57616	GCCACCAATCAGATCTTACA	0
OFF1-gRNA1	20:+44624674	GCCACCAATCAGATCCTATA	2

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Table 1. Potential off-target sites of gRNA1 (mismatches are marked in red)

Target sites and potential off-target sites	Locus	Target and off-target sequences (5'–>3')	Num of mismatches
[ ]	2:-57571	TCAGGGGGATGGGATCTATG	0

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Table 2. Potential off-target sites of gRNA2 (mismatches are marked in red)

E. Description of the screening tests to confirm the targeted gene editing of Aux/IAA gene

Specific primers will be designed to amplify the [ ] gene from T0 events, DNA bands of interest (wild-type size and mutated size) will be cut out of the agarose gel and purified. Both DNA samples will be sequenced by Sanger to identify the deleted region in T0 plants. The events that present the desired deletion in the target site will be selected and harvested. T1 plants will be identified as described above to select the positive ones that carry the [ ] deletion.

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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 auxinics inhibitors family in T2 plants will be performed.

Primer Name	Sequence	Tm	Product size
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	
usAtU6.2_Fw	GCTTGAATTCTTGCTTAGGAGTG	54.1°C	180 bp / 403 bp
scaffold_Rv	CCACTTTTTCAAGTTGATAACGGACTAGC	58 °C	
GmUBI3_Fw	CTCCTCCTTCCTTCGCAGTTCAATTC	59.3 °C	578 bp
GmUBI3_Rv	ACCCTAATCCACGGCAATAGAATCAAC	58.7 °C	
Cas9-SPG.1_Fw	ACCAAGGCTCCACTCTCAGCTTCTAT	61,1ºC	525 bp
Cas9-SPG.1_Rv	ACTAGCACCCTTATCCACCACCTCTT	61ºC	
Cas9-SPG.2_Fw	CAGAAAGGTGACCGTGAAGCAGC	60.5ºC	489bp
Cas9-SPG.2_Rv	CTATCACCCTGTCCTGAAACCTGAGC	60,6ºC	
Cas9-SPG.3_Fw	ACAGGTGAGATCGTGTGGGATAA	57.6 °C	522 bp
Cas9-SPG.3_Rv	GTAGTGAGAAGCGAGGTAAAGAAAG	55.1 °C	
SV40_Fw	CGACTCTGGTGGTTCTCCAAAGAAA	59ºC	476 bp
tNOS_Rv	TGATAATCATCGCAAGACCGGCAAC	59,5ºC	
RBtDNA_Fw	TGACAGGATATATTGGCGGGTAAACC	58,4ºC	643 bp
pVS1StaA_Rv	CCATATGTCGTAAGGGCTTGGC	58,1ºC	

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

F. Description of the trait

The trait introduced by gene editing is herbicide resistance. The State University of Colorado (USA) protects a mutation (deletion of 9 amino acids) of the *SoIAA2* gene of *Sisymbrium orientale*, which confers resistance to different auxinic herbicides[1]. The gene targeted from AUX/IAA family [ ] encodes for an auxin co-receptor AUX/IAA (auxin/indole-3-acetic acids), an auxin-associated transcriptional repressor. Auxin plays a central role in many aspects of plant growth and development. Auxin-regulated control of gene expression is mediated by proteins belonging to three families that include receptors (F-box proteins); transcriptional repressors (Aux/IAA) and auxin response factors (ARF). Auxin signal transmission depends on interactions between components of these families of proteins. When auxin levels are low in the nucleus, Aux/IAA proteins inhibit ARF activity due to an heterodimerization with ARF, blocking auxin-dependent gene expression. When auxin levels increase, auxin unblocks ARFs by promoting binding between the Aux/IAA and TIR1/AFB proteins that together form the auxin co-receptor complex. TIR/AFB proteins are substrate-binding subunits of E3 ubiquitin ligases. The formation of this complex triggers the polyubiquitination of Aux/IAA, followed by its degradation[2]. Synthetic auxin herbicides, such as 2,4-D, mimic the effects of natural auxins and induce strong changes in gene expression that

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ultimately lead to lethal responses in plant growth[1]. BioHeuris has an exclusive patent license from the State University of Colorado (USA) that protects a mutation (deletion of 9 amino acids) of the *SoIAA2* gene of *Sisymbrium orientale*, which confers resistance to different auxinic herbicides[1]. In soybean, this deletion is equivalent to [ ] amino acids, which could be specifically deleted by directing two CRISPR/Cas9 complexes to the target site, one guided by a gRNA upstream and the other by a gRNA downstream of said site. CBI Deleted

G. References

1. Figueiredo, M. R. A. de *et al.* An in-frame deletion mutation in the degron tail of auxin coreceptor *IAA2* confers resistance to the herbicide 2,4-D in *Sisymbrium orientale*. *Proc. Natl. Acad. Sci.* **119**, e2105819119 (2022).
2. Singh, V.K., Jain, M., 2015. Genome-wide survey and comprehensive expression profiling of Aux/IAA gene family in chickpea and soybean. Front. Plant Sci. 6. https://doi.org/10.3389/fpls.2015.00918
3. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337, 816–821. https://doi.org/10.1126/science.1225829
4. Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., Joung, J., van der Oost, J., Regev, A., Koonin, E.V., Zhang, F., 2015. Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. Cell 163, 759–771. https://doi.org/10.1016/j.cell.2015.09.038

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 *Aux/IAA* gene.**

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[ ]:Represents the [ ] CBI Deleted nucleótides deleted with the 2 gRNA designs.

ATG: Transcription start codon.

TGA: transcription termination codon.

2. Amino acid sequence corresponding to *Glycine max* Aux/IAA gene.

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[ ]: Represents the [ ] aminoácides deleted with the 2 gRNA designs. CBI Deleted

3. Full vector sequence

>pBH582

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Sets of primers that will be used to detect transgenic events

pVS1RepA_Fw: GCCGATGGCGGAAAGCAGAAAG
pVS1oriV_Rv: ACGGGTTCCGGTTCCCAATGTA
KanR_Fw: TACCTTAGCAGGAGACATTCCTTCCG
LBtDNA_Rv: GTTTACACCACAATATATCCTGCCACC
SpecR_Fw: ACATCCTTCGGCGCGATTTTG
SpecR_Rv: GCCTGAAGCCACACAGTGATATTG
usAtU6_Fw: GCTTGAATTCTTGCTTAGGAGTG
scaffold_Rv: CCACTTTTTCAAGTTGATAACGGACTAGC
GmUBI3_Fw: CTCCTCCTTCCTTCGCAGTTCAATTC
GmUBI3_Rv: ACCCTAATCCACGGCAATAGAATCAAC
Cas9-SPG.1_Fw: ACCAAGGCTCCACTCTCAGCTTCTAT
Cas9-SPG.1_Rv: ACTAGCACCCTTATCCACCACCTCTT
Cas9-SPG.2_Fw: CAGAAAGGTGACCGTGAAGCAGC

Cas9-SPG.2_Rv:	CTATCACCCTGTCCTGAAACCTGAGC
Cas9-SPG.3_Fw:	ACAGGTGAGATCGTGTGGGATAA
Cas9-SPG.3_Rv:	GTAGTGAGAAGCGAGGTAAAGAAAG
SV40_Fw:	CGACTCTGGTGGTTCTCCAAAGAAA
tNOS_Rv:	TGATAATCATCGCAAGACCGGCAAC
RBtDNA_Fw:	TGACAGGATATATTGGCGGGTAAACC
pVS1StaA_Rv:	CCATATGTCGTAAGGGCTTGGC