

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

**Confidential Business Information Deleted**

Dear Ms. Juarez,

Bioheuris Inc. respectfully requests a petition for determination on the regulatory status of a cotton (*Gossypium hirsutum*) that we intend to develop using Non-Homologous End-Joining (NHEJ), CRISPR-Cas9-mediated gene editing technology. The proposed cotton 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 cotton 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 cotton 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.  
1100 Corporate Square Dr., St. Louis, MO 63132  
Email: [carlos.perez@bioheuris.com](mailto: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**

#### D. 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)<sup>1</sup>. Studies of the resistant population progeny revealed that the resistance was inherited as a single dominant allele<sup>2</sup>. 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 degra tail (DT) of the auxin coreceptor Aux/IAA2 of *S. orientale* (SoIAA2)<sup>3</sup>. 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 [ ] 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.

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#### E. Second Comparator Plant

Centromere-mediated chromosome elimination (CCE) is a novel breeding method used to accelerate breeding in crops<sup>4</sup>. 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.

#### F. 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<sup>5</sup>. 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<sup>6</sup>. 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.

## G. Genotype of the Modified Plant

### a. Description of plant's genus, species

- Class: *Magnoliopsida*
- Family: *Malvaceae*
- Genus: *Gossypium*
- Species: *hirsutum*

### b. Description of reagents used for genome editing

BioHeuris intends to develop cotton (*Gossypium hirsutum*) using NHEJ (Non Homologous End Joining) technology, where the DNA vector that carries the editing machinery is introduced by transformation with *Agrobacterium* in the cotton explants from hypocotyls of an in vitro cotton 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<sup>3,4</sup>. 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.

Cotton tissues will be transformed with the corresponding DNA vector (described below) using *Agrobacterium*-mediated transformation followed by plant regeneration. The expected genotype in the cotton plant after gene editing is the deletion of [ ] nucleotides between the positions [ ] (CDS) of the Aux/IAA homoeolog genes [ ], which is equivalent to [18] amino acids and respects the amino acid identity of the rest of the proteins. The final product, obtained by this strategy will not contain any foreign DNA in the cotton genome, therefore will not constitute a new genetic combination.

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Steps involved to obtain the intended cotton plant:

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

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#### Vector features and coordinates:

- pVS1 StaA (stability protein from the Pseudomonas plasmid pVS1) (13483...14112)
  - pVS1 RepA (replication protein from the Pseudomonas plasmid pVS1) (351...1424)
  - pVS1 (OriV, Pseudomonas plasmid pVS1 replication origin ) (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) (13179...13203)
  - 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)
  - OsUbi (promoter for Oryza sativa polyubiquitin gene RUBQ2) (6632...8341)
  - Cas9-SpG (variant of Cas9 endonuclease from the Streptococcus pyogenes Type II CRISPR/Cas system with NGN PAM requirement) (8399...12502)
  - SV40 NLS (nuclear localization signal of SV40 (simian virus 40) large T antigen) (12515...12535)
  - CaMV poly(A) signal (cauliflower mosaic virus polyadenylation signal) (12605...12781)
2. Transformation of cotton callus mediated by *Agrobacterium tumefaciens* (LBA4404) carrying the vector (pBH581) through somatic embryogenesis.
  3. Spectinomycin selection in the transformed explants and transgenic plant regeneration (T0).
  4. T0 genotyping by Sanger sequencing of the targeted gene to confirm the intended mutation.

5. T1 seed harvest from edited events carrying the [ ]. CBI Deleted
6. 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.
7. Growth and development of T1 transgene-free edited plants and seeds harvest.
8. Herbicide tolerance assay to auxin 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 cotton plant after gene editing is the deletion of [ ] CBI Deleted nucleotides between the positions [ ] (CDS) of the Aux/IAA gene, which is CBI Deleted equivalent to [ ] amino acids and respects the amino acid identity of the rest of the proteins. CBI Deleted The gRNA sequences used to modify the [ ] CBI Deleted genes have been designed to only make this change in the amino acid sequence of CBI Deleted these two proteins. Additionally, off-target sites containing mismatches will be examined by Sanger sequencing at the corresponding position with specific primers. The potential off-target sites of gRNA1 and gRNA2 found using CRISPR RGEN tool software are listed in Table 1 and

Table 2 respectively. There were no potential off-targets for gRNA1, and only one with 2 mismatches for gRNA2. Edited plants in off-target sites will be discarded.

Target sites and potential off-target sites	Site	Position	Target and off-target sequences	Num of mismatches
[ ]	Target	4103350	GGCCACCGATCCGCTCTTAT	0
[ ]	Target	5051448	GGCCACCGATCCG <b>G</b> TCTTAT	1

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

Target sites and potential off-target sites	Site	Position	Target and off-target sequences	Num of mismatches
[ ]	Target	4103297	TCGGAGGGTGGTGGGATTTA	0
[ ]	Target	5051395	TC <b>T</b> GAGGGTGGTGGGATTTA	1
A02-OFF1	Off-target	230398	TC <b>T</b> GAGGGTGGT <b>C</b> GGATTTA	2

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

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

Specific primers will be designed to amplify the [ ] genes 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/s will be selected and harvested. T1 plants will be identified as described above to select the positive ones that carry the [ ] aminoacid deletion.

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To confirm there is no remaining exogenous vector DNA in edited cotton 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.

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

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

Primer Name	Sequence	Tm	Product size
pVS1RepA_Fw	GCCGATGGCGAAAGCAGAAAG	61.3 °C	655 bp

<b>pVS1oriV_Rv</b>	ACGGGTTCCGGTCCCAATGTA	60.8 °C	
<b>KanR_Fw</b>	TACCTTAGCAGGAGACATTCCCTCCG	59,6°C	542 bp
<b>LBtDNA_Rv</b>	GTTTACACCACAATATATCCTGCCACC	57,6°C	
<b>SpecR_Fw</b>	ACATCCTTCGGCGCGATTTTG	58,6 °C	499 bp
<b>SpecR_Rv</b>	GCCTGAAGCCACACAGTGATATTG	58.2 °C	
<b>usAtU6.2_Fw</b>	GCTTGAATTCTTGCTTAGGAGTG	54.1°C	180 bp / 403 bp
<b>scaffold_Rv</b>	CCACTTTTTCAAGTTGATAACGGACTAGC	58°C	
<b>OsUBI_Fw</b>	TCACTCAAAAAAAAAATCAACGAGACG	57°C	610 bp
<b>OsUBI_Rv</b>	CACCAATCACTCAGGCATGACT	59°C	
<b>Cas9-SPG.1_Fw</b>	ACCAAGGCTCCACTCTCAGCTTCTAT	61,1°C	525 bp
<b>Cas9-SPG.1_Rv</b>	ACTAGCACCTTATCCACCACCTCTT	61°C	
<b>Cas9-SPG.2_Fw</b>	CAGAAAGGTGACCGTGAAGCAGC	60.5°C	489bp
<b>Cas9-SPG.2_Rv</b>	CTATCACCTGTCCTGAAACCTGAGC	60,6°C	
<b>Cas9-SPG.3_Fw</b>	ACAGGTGAGATCGTGTGGGATAA	57.6°C	522 bp
<b>Cas9-SPG.3_Rv</b>	GTAGTGAGAAGCGAGGTAAGAAAAG	55.1°C	
<b>SV40_Fw</b>	AGAGGAAGGTTTGATACAAGTAACCG	58°C	450 bp
<b>tNOS_Rv</b>	TGATAATCATCGCAAGACCGGCAAC	59,5°C	
<b>RBtDNA_Fw</b>	TGACAGGATATATTGGCGGGTAAACC	58,4°C	643 bp
<b>pVS1StaA_Rv</b>	CCATATGTCGTAAGGGCTTGGC	58,1°C	

## H. Description of New 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 *So/IAA2* gene of *Sisymbrium orientale*, which confers resistance to different auxinic herbicides<sup>1</sup>. The genes targeted from AUX/IAA family [ ] CBI Deleted encodes for auxin co-receptors AUX/IAA (auxin/indole-3-acetic acids), auxin-associated transcriptional repressors. 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. TIR1/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<sup>2</sup>. Synthetic auxin herbicides, such as 2,4-D, mimic the effects of natural auxins and induce strong changes in gene expression that ultimately lead to lethal responses in plant growth<sup>1</sup>. 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<sup>1</sup>. In cotton, 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 the targeted sites.

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## I. 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.



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Sincerely Yours,

Dr. Carlos Perez

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## Section 1: Sequence

1. Nucleotide sequence corresponding to *Aux/IAA* gene [ ].

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s the [ ] nucleótides deleted with the 2 gRNA designs.

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**ATG**: Transcription start codon.

**TGA**: transcription termination codon.

2. Amino acid sequence corresponding to *Gossypium hirsutum* Aux/IAA gene [ ] CBI Deleted

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3. Nucleotide sequence corresponding to *Aux/IAA* gene [ ]. CBI Deleted

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s the [ ] nucleótides deleted with the 2 gRNA designs.

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ATG: Transcription start codon.

TGA: transcription termination codon.

**4. Amino acid sequence corresponding to *Gossypium hirsutum* Aux/IAA gene [ ]**

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designs.

**5. Full vector sequence**

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