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ZeaKal

August 7, 2023

Bernadette Juarez
U.S. Department of Agriculture
APHIS Deputy Administrator
Biotechnology Regulatory Services

Does Not Contain Confidential Business Information

RE: Regulatory Status Review (RSR) of transgenic soybean with increased oil and protein content in the seed-construct 2. RSR number 23-160-01rsr

Dear Ms. Juarez,

ZeaKal, Inc. respectfully requests a Regulatory Status Review (RSR) of our developed transgenic soybean line that contains four linked transgene cassettes incorporated into its genome, including a glufosinate selectable marker, and results in a soybean plant that has increased oil and protein content in the seed. As described below, soybean that has been genetically modified to contain this construct is unlikely to pose a plant pest risk and we request confirmation that it is not regulated under 7 CFR part 340[1].

In order to provide you with necessary details to evaluate this GE plant and its plant-trait-mechanism of action (MOA), we will describe this plant and plant-trait-MOA by following the guidelines presented in part 340.4(a)(4)(i-iii) as well as on pages 29808-29809 of 85 FR 29790-29838, Docket No. APHIS–2018–0034 and the Guide for Requesting a Regulatory Status Review under 7 CFR part 340 Document ID BRS-GC-2020-0003 issued December 20, 2022.

Description of the comparator plant(s), to include genus, species, and any relevant subspecies information

Soybean, *Glycine max*, L.

[1] On May 18th, 2020, USDA-APHIS revised its regulations at 7 CFR part 340 (85 FR 29790- 29838, Docket No. APHIS–2018–0034) with an effective date of April 5, 2021 for RSR processes of certain crops, including soybean, under Sections 340.4. Unless otherwise indicated, the references to “Part 340” herein are as of April 5, 2021.

Genotype of the modified plant, including a detailed description of the differences in genotype between the modified and unmodified plant

The modified plant contains [

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>ZeaKal T-DNA-2

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To further describe the T-DNA insertion, a description of the genetic elements of the T-DNA is included in Table 1.

Table 1. Description of genetic elements present in the T-DNA of soybean plant.

Nucleotide position	Genetic Element	Source Organism	Function
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Amino acid sequences and alignments of the native and ZeaKal amino acid sequences are shown below and highlight the modifications compared to the native sequence. As noted in Table 1, [

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The [ ] amino acid sequence is show below and [CBI-Deleted

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The [ ] amino acid sequence is shown below. [CBI-Deleted

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Detailed description of the new trait(s) of the modified plant.

Intended traits & phenotypes

There are three intended traits of ZeaKal's GE soybean. Two of these traits have been previously determined by APHIS not to require regulation under 7 CFR part 340 in soybean and can be found on BRS' Plant-Trait-MOA table. These two traits include:

(i) A selectable marker PAT (phosphinothricin N-acetyltransferase) which confers resistance to glufosinate by catalyzing the conversion of L-phosphinothricin (L-PPT) to a non-phytotoxic form (N-acetylphosphinothricin) (reference numbers: 96-068-01p, 98-014-01p, 98-238-01p, 09-349-01p, 11-234-01p, 12-215-01p, 12-272-01p, 17-223-01p).

(ii) A trait that increases seed oil and protein content by co-expression of an oil synthesizing enzyme [ ] and an oil encapsulating CBI-Deleted

protein [ ] in green tissues resulting in elevated photosynthetic capacity (reference number: 21-117-01rsr). CBI-Deleted

The third trait alters (increases) the seed oil content by expression of an oil synthesizing enzyme [ ] in the seed resulting in increased seed oil content. CBI-Deleted

The three traits combined result in a soybean plant with a phenotype that is resistant to the herbicide glufosinate, has elevated photosynthetic capacity, and has increased oil and protein content in the seed.

Description of the Mechanism(s) of Action (MOA)

The Plant-Trait MOAs of ZeaKal's GE soybean can be found in Table 2. As stated above, two plant-trait MOAs have been previously determined by APHIS not to require regulation under 7 CFR part 340. The third trait involves seed-specific expression of an oil synthesizing enzyme [ ]. Each MOA is further described below: CBI-Deleted

Selectable Marker (PAT)

Phosphinothricin N-acetyltransferase (PAT) which confers resistance to glufosinate by catalyzing the conversion of L-phosphinothricin (L-PPT) to a non-phytotoxic form (N-acetylphosphinothricin) was used as a selectable marker during transformation of ZeaKal's GE soybean and was previously determined by APHIS not to require regulation under 7 CFR part 340 (reference numbers: 96-068-01p, 98-014-01p, 98-238-01p, 09-349-01p, 11-234-01p, 12-215-01p, 12-272-01p, 17-223-01p).

The active ingredient in the herbicide glufosinate ammonium is L-phosphinothricin (L-PPT). L-PPT (also known as the amino acid, 4-[hydroxy-(methyl)phosphinoyl]-D,L-homoalanine) shares structural similarities with glutamic acid. The L-isomer (but not the D-isomer) inhibits glutamine synthetase (GS) by competitively binding to the substrate (glutamate/glutamic acid) binding site. GS is required for glutamine synthesis and to detoxify ammonia build up in cells. (OECD, 1999; Thompson et al., 1987; Center for Environmental Risk Assessment, 2011.) The PAT enzyme utilizes acetyl-coA as a co-substrate to acetylate L-PPT at the N-terminus and results in a compound (N-acetylphosphinothricin) with no herbicidal activity (i.e., no inhibition of GS). In this respect, the MOA of PAT is to modify the active ingredient in glufosinate (L-PPT) into an inactive form (N-acetylphosphinothricin). In ZeaKal's soybean plant, the PAT protein is under the control of a [CBI-Deleted

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Elevated Photosynthesis and Increased Oil and Protein Content in the Seed

The second MOA in ZeaKal's GE soybean has also previously determined by APHIS not to require regulation under 7 CFR part 340 (reference number: 21-117-01rsr). The co-expression of an oil synthesizing enzyme [ ] and an oil encapsulating protein CBI-Deleted
[ ] in green tissues results in elevated photosynthesis. CBI-Deleted

This phenotype is accomplished through the co-expression of two plant genes under the control of green tissue specific promoters to produce [ ]. The production and CBI-Deleted
accumulation of [
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] Over the course of the growing season, the elevated photosynthesis results in the fixation of additional carbon which manifests as increased seed oil content. The elevated photosynthesis also supports higher nitrogen fixation by rhizobia which is seen as increased protein content in the seed.

The two plant genes co-expressed include [
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Oil Synthesizing Enzyme Expressed in Seeds

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Table 2. Trait, Phenotype and MOAs of ZeaKal's transgenic soybean.

Plant	Scientific Name	Trait	Phenotype	Mechanism of Action
Soybean	*Glycine max*	Marker gene (herbicide resistance)	Resistance to glufosinate	PAT (phosphinothricin N-acetyltransferase) confers resistance to glufosinate by catalyzing the conversion of L-phosphinothricin (L-PPT) to a non-phytotoxic form (N-acetylphosphinothricin)
Soybean	*Glycine max*	Altered seed composition	Increased seed oil and protein content	Co-expression of oil synthesizing enzyme and oil encapsulating protein in green tissues resulting in elevated photosynthesis
Soybean	*Glycine max*	Altered seed composition	Increased seed oil content	Seed-specific expression of an oil synthesizing enzyme resulting in increased seed oil content

Additional data

Over the past several years, ZeaKal has generated several dozen soybean events with different variations of the described molecular stacks. [

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]. These events have been grown in USDA-APHIS regulated field trials at multiple sites. Over 6 years and all 6 sites, we have not observed differences in the overall reproductive cycle or overall soybean plant morphology compared to the non-transgenic control plants. In addition, we have not observed any differences in the susceptibility to pests nor observed any harm to non-target organisms beneficial to agriculture, nor have we observed any evidence that the technology has increased the plants' ability to be a pest or act as a reservoir for a plant pest.

Thank you for taking the time to review this inquiry. I'm happy to address any questions you may have.

Sincerely,

Amy Curran, Ph.D.
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zeakal.com

References

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Center for Environmental Risk Assessment IRF. 2011. *A review of the environmental safety of the PAT protein*. Environ Biosafety Res 10 (2011) 73–101. DOI: 10.1051/ebr/2012004

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OECD (1999). Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide. Organization for economic Cooperation and Development (OECD), Paris, France. ENV/JM/MONO(99)13.

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Thompson C. J., Movva N. R., Tizard R., Crameri R, Davies J. E., Lauwereys M., and Botterman J. (1987). Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. The EMBO Journal 6(9):2519−2523.

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