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Animal and Plant Health Inspection Service
United States Department of Agriculture
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Dr. Firko:

CONTAINS CONFIDENTIAL BUSINESS INFORMATION

Re: Confirmation of Regulatory Status of Nutritionally-Enhanced Wheat Developed by TALEN® Technology

Calyxt (formerly Cellectis Plant Sciences) is developing technology that enables precise genome modification in economically important crops used for food and feed. One of the products that Calyxt is focused on is a wheat product (*Triticum aestivum*) with increased nutritional value, [ ]KO Wheat, CBI-deleted
defined as a null-segregant of wheat lines created by the transient expression of the construct described below. [ ]KO Wheat has [ ] attributable to the knockout of the [CBI-deleted
] gene achieved through transient expression of a Transcription Activator-Like Effector Nuclease CBI-deleted
(TALEN®). The [ ]KO Wheat was developed using a similar method as described in the Regulated Letter CBI-deleted
of Inquiry for the development of the FAD2KO Soybean, FAD3KO Soybean, and MLO_KO Wheat, which were submitted by Cellectis Plant Sciences/Calyxt to USDA/APHIS on November 17, 2014, May 21, 2015, and August 25, 2015, respectively.

Wheat is not a plant pest or an invasive species, the genetic elements used to generate [ ]KO Wheat CBI-deleted
are sourced from fully classified organisms, and the genomic modification process does not introduce any plant pest DNA components. There is no scientifically valid basis for concluding that [ ]KO Wheat is, or CBI-deleted
will become, a plant pest within the meaning of the Plant Protection Act. Calyxt therefore asserts that under current regulations, [ ]KO Wheat is not a regulated article within the meaning of 7 CFR §340.1 CBI-deleted
because it does not satisfy the criteria that would subject it to oversight of the USDA's Animal and Plant Health Inspection Service (APHIS).

It is Calyxt's intention to potentially commercialize the [ ]KO Wheat. This would include, but not be CBI-deleted
limited to, seed and grain production that would require both interstate movement and unconfined environmental release. Before proceeding Calyxt respectfully requests confirmation from USDA-APHIS' Biotechnology Regulatory Services of the regulatory status of [ ]KO Wheat developed using CBI-deleted
TALEN® genome editing technology. If the agency does not concur with Calyxt's interpretation of the current regulations, Calyxt requests that the Agency provide us with its scientific rationale for concluding that [ ]KO Wheat is or will become a plant pest. CBI-deleted

I. Transformation Background

To further assist APHIS in understanding the origin of [ ]KO Wheat, a summary of information on the recipient plant, genetic elements, and process used to modify the recipient plant's genomic DNA, is provided below. CBI-deleted

a. [ ]KO Wheat (*Triticum aestivum* L. subsp. *aestivum*) CBI-deleted

[ ]KO Wheat contains a single gene (multi-allele) knockout achieved through expression of a specially designed TALEN®. TALEN® technology is a relatively new method of targeted mutagenesis that is functionally equivalent to other methods of achieving targeted deletions such as meganucleases, zinc-finger nucleases, and CRISPR/Cas9. These methods rely on customizable DNA recognition sequences coupled to site-specific nucleases that create double stranded breaks in genomic DNA. Following the introduction of a double stranded break, the cells' natural DNA repair mechanism completes the repair by non-homologous end-joining (NHEJ). DNA repair via NHEJ occasionally produces small disruptions in the targeted gene that lead to frameshift mutations and disruption of the targeted protein function. In this way, TALEN® technology can achieve a targeted gene knockout that produces a desirable phenotype. CBI-deleted

In [ ]KO Wheat, a specially designed TALEN® expression cassette and a selectable marker expression cassette was co-introduced by particle bombardment transformation of wheat immature embryos. A selectable marker system was used to preferentially identify or concentrate wheat explants with the desired integration of the TALEN® expression cassette and the selectable marker expression cassette. The TALEN® reagent was expressed in the wheat cells to achieve the targeted gene knockouts via the deletion of nucleotides, resulting in a gene disruption. Standard tissue culture techniques were used to regenerate callus and subsequently whole plants. Polymerase Chain Reaction (PCR) techniques were then used to screen regenerated wheat plants to identify transgenic plants that also contained target-site disruptions of alleles of the Wheat [ ] gene. Table 1 describes the genetic elements used to produce the intended phenotype in [ ]KO Wheat. CBI-deleted CBI-deleted CBI-deleted

b. Recipient Wheat (*Triticum aestivum* L. subsp. *aestivum*)

Wheat is not a federal noxious weed. It is a cereal grain in the Poaceae family that is grown worldwide; it is responsible for providing approximately 20% of the daily protein and food calories in the human diet. It is the world's most widely grown crop by area and third most produced cereal following maize and rice. Wheat is thought to be the first cereal domesticated, with the center of origin reported as the Fertile Crescent region of the Middle East, specifically what is present-day southeastern Turkey. In the United States, wheat is produced in almost all 50 states, although commercial production is concentrated in approximately 15 states mostly in the central/northern plains and Pacific Northwest. Wheat is a hexaploid species with 42 chromosomes; a draft genome sequence of bread wheat was published in 2014.

Table 1. Genetic elements used for targeted gene knockout in [ ]KO Wheat

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Genetic Element	Source	Function	
[ ]	[ ]	Promoter to regulate transcription of the TALEN® reagent.	CBI-deleted
TAL effector	*Xanthomonas* spp.	An array of 32-amino acid DNA-binding motifs that specifically recognize target sequences within the [ ]. The TAL effector binding domain is exclusive of all native sequences responsible for cell infection and pathogenicity: nuclear localization signal peptide (NLS) and acidic transcription activation domain (AAD).	CBI-deleted
FokI	*Flavobacterium okeanokoites*	A bacterial type IIS restriction endonuclease consisting of an N-terminal DNA-binding domain and a non-specific DNA cleavage domain at the C-terminal that cleaves wheat genomic DNA downstream of the TALEN® binding domain.	
[ ]	[ ]	Sequence to regulate production of an mRNA of the TALEN® reagent.	CBI-deleted
[ ]	[ ]	Promoter to regulate transcription of the selectable marker gene.	CBI-deleted
[ ]	[ ]	Selectable marker to confer resistance to the plant herbicide [ ]	CBI-deleted
[ ]	[ ]	Sequence to regulate production of an mRNA of the [ ] selectable marker.	CBI-deleted
[ ]	[ ]	Promoter to regulate transcription of the reporter gene.	CBI-deleted
[ ]	[ ]	Reporter marker for identification of transgenic plants	CBI-deleted
[ ]	[ ]	Sequence to regulate production of an mRNA of the [ ] reporter gene.	CBI-deleted

II. APHIS' Jurisdiction

APHIS defines a "regulated article" as:

> Any organism which has been altered or produced through genetic engineering, if the donor organism, recipient organism, or vector or vector agent belongs to any genera

> or taxa designated in §340.2 and meets the definition of plant pest, or is an unclassified organism and/or an organism whose classification is unknown, or any product which contains such an organism, or any other organism or product altered or produced through genetic engineering which the Administrator, determines is a plant pest or has reason to believe is a plant pest. Excluded are recipient microorganisms which are not plant pests and which have resulted from the addition of genetic material from a donor organism where the material is well characterized and contains only non-coding regulatory regions.

Consistent with the PPA's definition of a plant pest, APHIS further defines a "plant pest" as:

> Any living stage (including active and dormant forms) of insects, mites, nematodes, slugs, snails, protozoa, or other invertebrate animals, bacteria, fungi, other parasitic plants or reproductive parts thereof; viruses; or any infectious agents or substances, which can directly or indirectly injure or cause disease or damage in or to any plants or parts thereof, or any processed, manufactured, or other products of plants.

APHIS further claims that its regulations are consistent with the Coordinated Framework, because they apply "only [to] genetically engineered organisms or products which are plant pests or for which there is reason to believe are plant pest, and not to... an organism or product merely because of the process by which it was produced. APHIS has further stated that its concern arises only "when an organism or product is altered or produced by genetic engineering and one or more of its constituents (donor, vector/vector agent or recipient) comes from a family or genus of organisms know to contain plant pests... This is because... there is a risk that certain undesirable traits may be transferred to the new organism and may survive when the organism is released into the environments."

Under APHIS regulations, an organism is considered a "regulated article" "if the donor organism, recipient organism, or vector or vector agent belongs to a genera or taxa designated in 7 CFR §340.2, *and* the organism meets the definition of a plant pest." The language of the regulation requires that both criteria must be met to satisfy the definition of a regulated article.

The TALEN® reagent used for targeted mutagenesis of [ ]KO Wheat contains a highly derivatized DNA-binding domain from *Xanthamonas*, a taxa designated in 7 CFR §340.2. The DNA-binding domain consists of an array of a 32-amino acid highly conserved sequence except for the hypervariable amino acid residues at positions 12 and 13 called repeat-variable di-residues (RVDs). Unlike the TAL effectors found in *Xanthamonas*, which are capable of infecting plants, the coding sequences necessary for infection and pathogenicity, the nuclear localization signal peptide (NLS) and the acidic transcription activation domain (AAD), are removed from TALEN® reagents, leaving only the DNA-binding domain. The TALEN® is customized to recognize the DNA target sequence of the recipient plant, in this case, the second exon of the [ ] gene of wheat. CBI-deleted CBI-deleted

Another definition of a "regulated article" includes "any product which contains such an organism [i.e., an organism that is or contains DNA sequences from a plant pest]." [CBI-deleted
]KO Wheat does not meet that definition because it no longer contains DNA sequences from a plant pest or pathogen. [ ]KO Wheat is a null segregant of a wheat transgenic CBI-deleted
producer plant in which the TALEN® reagent was expressed from a stably integrated expression cassette. In the transgenic producer plant, molecular analyses confirmed the presence of the TALEN® expression cassette and the selectable marker expression cassette, as well as the targeted disruption of the [ ] gene. Subsequent self-pollination CBI-deleted
of the transgenic producer plant resulted in the [ ]KO Wheat line, which no longer CBI-deleted
contained the TALEN® expression cassette or the selectable marker expression cassette, but retained the targeted disruption of alleles of the [ ] gene. Furthermore, PCR CBI-deleted
analysis confirms the absence of TALEN®-derived DNA or retention of any components of the either of the expression cassettes in the genome of [ ]KO Wheat. Therefore, [CBI-deleted
]KO Wheat does not satisfy this criterion to qualify as a "regulated article." CBI-deleted

Another definition of a "regulated article" includes organisms that are unclassified or whose classification is unknown. The introduced trait provides wheat varieties with
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III. Finding that [ ]KO Wheat is Not a Regulated Article is Consistent With Previous APHIS Determinations CBI-deleted

APHIS has made a number of determinations that plants with specific targeted mutations are not "regulated articles," including certain plants containing a targeted gene knock-out by zinc-finger nucleases or meganucleases. For example, APHIS determined that "GE plants containing targeted deletions, caused by naturally-occurring DNA repair after the targeted break is made by zinc-finger nuclease, and *in which no genetic material is inserted into the plant genome*, are not regulated articles under CFR part 340 [provided that] the nucleases used are not from a plant pest and no plant pest sequences are inserted into the plant genome" (Gregoire to Dow AgroSciences, Mar 8, 2012). APHIS also determined that certain plants containing "targeted gene deletions, caused by naturally-occurring DNA repair after the break is made by the I-CreI meganuclease... [wherein] no genetic material is inserted into the plant genome... will not, in most cases, be regulated articles under 7 CFR part 340" (Gregoire to Cellectis Plant Sciences, Dec 16, 2011).

APHIS determined that null segregant plants derived from genetically engineered plants are not "regulated articles." For example, APHIS determined that null segregants derived from a stably transformed sorghum species in which an RNAi construct containing plant pest sequences introduced by *Agrobacterium tumefaciens*-mediated transformation, are not regulated articles, whereas "the GE parent plants are regulated articles because a plant pest vector was used to introduce DNA that contains plant pest sequences" (Gregoire to University

of Nebraska-Lincoln, Jun 6, 2012). APHIS also determined that null segregants derived from stably transformed tobacco species in which a gene expression construct containing plant pest sequences introduced by *Agrobacterium tumefaciens*-mediated transformation, are not regulated articles (Gregoire to North Carolina State University, Oct 27, 2011). In these examples and in the case of the [ ]KO Wheat, null segregant plants contain no inserted foreign DNA, which is confirmed by sensitivity to herbicide application and molecular analyses. CBI-deleted

APHIS has also made several determinations that null-segregants derived from genetically engineered plants that also carry TALEN®-mediated gene disruptions are not "regulated articles". Examples include the FAD2KO Soybean, FAD3KO Soybean, and MLO Wheat products from Cellectis Plant Sciences (now Calyxt). Other examples of null segregants that are not considered "regulated articles" are also posted on USDA's website.

IV. Summary of Conclusions

In summary, wheat is not itself a plant pest, no plant pest elements are contained in [ ]KO Wheat, and all organisms involved in targeted mutagenesis of wheat are fully classified. Therefore, there is no scientifically valid basis to determine that [ ]KO Wheat is or will become a plant pest within the meaning of the Plant Protection Act. CBI-deleted CBI-deleted

Thank you for your consideration of this request. We look forward to receiving your response.

Sincerely,

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