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Biotechnology Regulatory Service
Animal and Plant Health Inspection Service
United States Department of Agriculture
4700 River Road, Unit 98
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Mr. Firko:

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Re: Confirmation that PPO_KO Potato is not a regulated article

Calyxt, Inc. (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 potato product (*Solanum tuberosum* L.), PPO_KO Potato, defined as potato lines created by the transient expression of the construct described below. PPO_KO Potato has improved processing characteristics attributable to the knockout (KO) of the Polyphenol Oxidase (PPO) gene achieved through transient expression of a Transcription Activator-Like Effector Nuclease (TALEN®). The PPO_KO Potato was developed using a similar method as described in the Regulated Letter of Inquiry for the development of the VInvKO Potato, which was submitted by Cellectis Plant Sciences to USDA/APHIS on July 29, 2013.

Potato is not a plant pest or an invasive species, the genetic elements used to generate PPO_KO Potato 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 PPO_KO Potato is, or will become, a plant pest within the meaning of the Plant Protection Act. Calyxt, Inc. therefore asserts that under current regulations, PPO_KO Potato is not a regulated article within the meaning of 7 CFR §340.1 because it does not satisfy the criteria that would subject it to oversight of the USDA's Animal and Plant Health Inspection Service (APHIS).

Before proceeding with further product development, Calyxt, Inc. requests that USDA/APHIS confirm that PPO_KO Potato, modified without incorporating any plant pest elements (as described more fully in Table 1 below), is not be considered a regulated article within the meaning of the current regulations. If the agency does not concur with Calyxt, Inc.'s interpretation of the current regulations, Calyxt, Inc. requests that the Agency provide us with its scientific rationale for concluding that PPO_KO Potato is or will become a plant pest.

I. Transformation Background

To further assist APHIS in understanding the origin of PPO_KO Potato, a summary of information on the recipient plant, genetic elements, and process used to modify the recipient plant's genomic DNA, is provided below.

a. PPO_KO Potato (*Solanum tuberosum* L.)

PPO_KO Potato contains a single gene (multi-allele) knockout achieved through transient expression of a specially designed TALEN®. TALEN® technology is a relatively new method of targeted mutagenesis that is functionally equivalent to other methods, such as meganucleases and zinc-finger nucleases, for achieving targeted deletions. These methods rely on customizable DNA recognition sequences coupled to sequence-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.

In PPO_KO Potato, a specially designed TALEN® reagent was introduced by polyethylene glycol (PEG)-mediated transformation of potato protoplasts. Upon introduction, the TALEN® reagent was transiently expressed in the protoplasts to achieve the targeted gene knockout. Protoplast cells were regenerated into calli and subsequently to whole plants. No selectable marker system was used to preferentially identify or concentrate potato cultures with the desired phenotype. Rather, Polymerase Chain Reaction (PCR) techniques were used to screen regenerated potato plants to confirm that no DNA from the TALEN® reagent remained in the plants selected for advancement. Table 1 describes the genetic elements used to produce the intended product quality phenotype in PPO_KO Potato.

b. Recipient Potato (*Solanum tuberosum* L.)

Potato is not a federal noxious weed. It is a starchy, tuberous crop of the Solanaceae family, which is grown worldwide as a source of calories for human and animal nutrition. It is the world's fourth-largest food crop behind rice, wheat, and maize. The center of origin of potato is reportedly present-day southern Peru and extreme northwestern Bolivia, although wild potato species occur throughout the Americas from the United States to southern Chile. In the United States, potatoes can be grown in all 50 states, although commercial production is concentrated in 10 states north of latitude 45° N (except the San Luis Valley of Colorado at 37° N and 7,500 feet above sea level). Potato is a cool season, herbaceous perennial that readily adapts to diverse climates wherever cool temperatures and abundant moisture allow plants to gather sufficient water to form the starchy tubers. Potato is a tetraploid with 48 chromosomes. The complete genome sequence of potato was published in 2011.

Table 1. Genetic elements used for targeted gene knockout in PPO_KO Potato

Genetic Element	Source	Function
[ ]	[ ]	Promoter to regulate transcription of the TALEN® reagent.

TAL effector	Xanthomonas spp.	An array of 34-amino acid DNA-binding motifs that specifically recognize target sequences within the first exon of the polyphenol oxidase (PPO) gene. 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).
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 potato genomic DNA downstream of the TALEN® binding domain.
[ ]	[ ]	Polyadenylation signal sequence to regulate production of an mRNA of the TALEN® reagent.

II. APHIS' Interpretation of Its 7 CFR §340 Regulations Dictates a Finding that PPO_KO Potato is Not a Regulated Article

a. APHIS Has Been Clear That Not All Genetically Modified Plants Are Subject to Regulatory Oversight

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."

b. PPO_KO Potato Does Not Fall Within the Regulatory Definition of a "Regulated Article"

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 PPO_KO Potato 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 34-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 first exon of the PPO gene of potato.

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]. PPO_KO Potato is a null segregant of potato protoplasts in which the TALEN® reagent was transiently expressed after delivery by PEG-mediated transformation. Phenotypic and DNA sequence analysis of the target gene confirms the TALEN® reagent was transiently expressed in PPO_KO Potato resulting in targeted deletions. Furthermore, PCR analysis confirms the absence of TALEN®-derived DNA or integration of the expression plasmid into the genome of PPO_KO Potato. Therefore, PPO_KO Potato does not satisfy this criterion to qualify as a "regulated article."

Another definition of a "regulated article" includes organisms that are unclassified or whose classification is unknown. The introduced trait enhances processing and attributes of potato by functional deletion of a native gene responsible for the enzymatic browning of many fruits and vegetables upon processing and/or damage. PPO catalyzes the conversion of monophenols to o-diphenols and o-dihydroxyphenols to o-quinones. The quinone products then react with amino acids, resulting in black or brown pigment deposits. It does not change the potato's basic biology or produce a

plant that would directly feed on, infect, parasitize, or contaminate plants, or adversely affect other organisms that are beneficial to plants.

III. Finding that PPO_KO Potato is Not a Regulated Article is Consistent With Previous APHIS Determinations

APHIS has made a number of determinations that genetically modified plants 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) (emphasis added). 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 also 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, null segregant plants contain no inserted DNA, which is confirmed by phenotypic and molecular analyses. Other examples are also posted on USDA's website.

IV. Summary of Conclusions

In summary, potato is not itself a plant pest, no plant pest elements are contained in PPO_KO Potato, and all organisms involved in targeted mutagenesis of potato are fully classified. Therefore, there is no scientifically valid basis to determine that PPO_KO Potato is or will become a plant pest within the meaning of the Plant Protection Act.

Thank you for your consideration and prompt confirmation of Calyxt's position that PPO_KO Potato is not a "regulated article" for the reasons stated above. We look forward to receiving your response.

Sincerely,

William Haun, PhD
Director of Product Development