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16 June, 2020

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737

Re: Confirmation of Regulatory Status of CRISPR-Cas9 Corn with Improved Yield.

Dear Ms. Juarez,

Corteva Agriscience respectfully requests confirmation from the USDA-APHIS's Biotechnology Regulatory Services (BRS) regarding the regulatory status of two maize lines developed using CRISPR-Cas9 gene editing technology to introduce targeted base pair substitutions (edits) in the FASCIATED EAR3 *(FEA3-2)* gene and that are designed to result in plants with increased grain yield. As described below, we do not consider the final plant lines to be the "regulated articles" subject to APHIS oversight under 7 C.F.R. Part 340[1] with respect to introduction into and/or through the U.S. because they do not contain any inserted genetic material from (i) a donor organism, recipient organism, or vector or vector agent listed in Section 340.2 and meeting the definition of "plant pest," or (ii) an unclassified organism and/or an organism whose classification is unknown. Nor is there a basis to believe that these maize lines are or will become a plant pest within the meaning of the Plant Protection Act. Moreover, the amino acid substitutions (edits) in the *(FEA3-2)* gene could have been achieved using conventional breeding techniques and therefore, the final plants lines are indistinguishable from maize plants that could have resulted from native genome variability or be developed in a conventional breeding program.

The maize *FEA3* gene encodes a leucine-rich-repeat (LRR) receptor expressed in the shoot apical meristem. Several *fea3* mutant alleles (*fea3-1 – fea3-4*) were obtained through ethyl methanesulfonate (EMS) mutagenesis and shown to enhance yield components in hybrid maize, such as ear length, kernel row number, kernel numbers per ear and ear weight (Je et al., 2016). We modified two maize lines to contain one of the weak mutant alleles, *fea3-2*, through

[1] On May 18th, 2020, USDA revised its regulations at 7 C.F.R. part 340 with an effective date of August 17, 2020 (85 FR 29790-29838, Docket No. APHIS–2018–0034), with the exception of permitting and RSR processes under Sections 340.4 and 340.5, which are effective April 5, 2021. Unless otherwise indicated, the references to "Part 340" herein are as of June 16, 2020.

gene editing to improve certain maize ear traits and yield. The *fea3-2* mutant allele contains a single amino acid substitution, Cysteine (C) to Tyrosine (Y), at position 417 (C417Y). CRISPR-Cas9 gene editing was used to introduce a targeted base pair substitution (edit) leading to the C417Y substitution into two maize inbred lines using the "gene edit" (SDN2) technique (Podevin et al., 2013). The DNA double strand break (DSB) was repaired in the presence of the supplied DNA repair template. The DNA repair template consisted of the sequence homologous to the targeted sequence near the DSB except for the specific base pair substitutions and flanked on each side by homology arms (sequences identical to the sequences immediately upstream and downstream of the genomic sequence to be edited). The C417Y substitution was accomplished by changing G in the Cysteine coding triplet (TGC) to A in the Tyrosine coding triplet (TAC). Additionally, three more base pair substitutions were introduced into the DNA repair template sequence. This is a typical approach to protect the DNA repair template from being cut by Cas9 as its sequence would otherwise be identical to the sgRNA target sequence[2]. These additional base pair substitutions were intentionally designed to result in silent mutations – i.e., mutations that do not cause an amino acid substitution. Homology arms induced the homology-directed recombination (HDR) DNA repair and resulted in repairing the DSB in a desired manner.

The figure below depicts targeted base pair substitutions made to generate the *fea3-2* allele.

Figure. Targeted base pair substitutions in the protein coding sequence to generate the fea3-2 allele. TGC to TAC change results in changing Cysteine to Tyrosine. T, A, and C in red font are additional base pair substitutions to protect the DNA repair template from cutting; these are silent mutations. Protospacer Adjacent Motif (PAM) sequence, immediately following the DNA sequence targeted by the Cas9 nuclease, is shown in box.

Additional Information on the Edited Lines:

Taxonomic description of organism
Maize, *Zea mays,* L.

Description of intended phenotype(s)
Plants with increased grain yield.

Description of intended activity (movement or release)
Field trials to evaluate the efficacy of introduced mutations.

[2] https://horizondiscovery.com/en/applications/crispr-cas9/homology-directed-repair-with-a-dna-donor-oligo

Description of intended genetic change in final product
Targeted base pair substitutions resulting in one amino acid substitution, as described above.

Description of vector or vector agent
Biolistic transformation with five plasmids (Plasmid 1 – Plasmid 5) and a double stranded (ds) oligonucleotide (Ds Oligo) serving as the DNA repair template.

Description of constructs
Elements of the CRISPR-Cas9 technology are indicated by bold font.

Plasmid 1		
Genetic Element	**Origin**	**Function**
ATTL1	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
UBI1ZM PRO	Maize *(Zea mays)*	Ubiquitin promoter.
UBI1ZM 5UTR (PHI)	Maize *(Zea mays)*	5' untranslated region of the ubiquitin promoter.
UBI1ZM INTRON1	Maize *(Zea mays)*	Intron from the ubiquitin promoter.
NPTII	*Escherichia coli*	Neomycin phosphotransferase gene conferring resistance to aminoglycoside antibiotics. Used as selectable marker in plant transformation.
PINII TERM	Potato *(Solanum tuberosum)*	Proteinase inhibitor II gene terminator to terminate transcription of the NPTII gene.
ATTL2	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
KAN	*Escherichia coli*	Kanamycin resistance gene to facilitate identification of the plasmid-containing bacterial clones during the vector construction process.
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.

Plasmid 2		
Genetic Element	**Origin**	**Function**
ATTL1	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
ZM-PLTP PRO	Maize (*Zea mays*)	Phospholipid transfer protein gene promoter to control expression of ZM-ODP2 coding sequence.
ZM-PLTP 5’ UTR	Maize (*Zea mays*)	Phospholipid transfer protein gene 5’ untranslated region for optimized expression of the ZM-ODP2 coding sequence.
ZM-ODP2 (ALT1)	Maize *(Zea mays)*	Alternate version of coding sequence of the ovule development protein 2 gene to improve regeneration frequencies.
OS-T28 TERM	Rice (*Oryza sativa)*	3'UTR and intergenic region of convergent gene pair LOC_Os03g60090.1 and LOC_Os03g60080.1

		to terminate transcription of the ZM-ODP2 coding sequence.
ATTL2	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
KAN	*Escherichia coli*	Kanamycin resistance gene to facilitate identification of the plasmid-containing bacterial clones during the vector construction process.
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.

Plasmid 3		
Genetic Element	**Origin**	**Function**
ATTL4	*Escherichia coli*	Gateway™ cloning system recombination site to facilitate vector construction process.
ZM-AXIG1 1.3KB PRO	Maize (*Zea mays*)	Auxin inducible gene 1 promoter to control expression of ZM-WUS2 (ALT1) coding sequence.
ZM-WUS2 (ALT1)	*Maize (Zea mays)*	Alternate version of the coding sequence of the wuschel 2 gene to improve regeneration frequencies.
IN2-1 TERM	*Maize (Zea mays)*	IN2 coding sequence terminator
AT-5-IV-2 INS	*Arabidopsis thaliana*	Putative insulator sequence to limit ZM-WUS2 (ALT1) transcript read-through.
ATTR1	*Escherichia coli*	Gateway™ cloning system recombination site to facilitate vector construction process.
KAN	*Escherichia coli*	Kanamycin resistance gene to facilitate identification of the plasmid-containing bacterial clones during the vector construction process.
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.

Plasmid 4		
Genetic Element	**Origin**	**Function**
ATTL2	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
KAN	*Escherichia coli*	Kanamycin resistance gene to facilitate identification of the plasmid-containing bacterial clones during the vector construction process.
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.
ATTL1	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
UBI1ZM PRO	Maize *(Zea mays)*	Ubiquitin promoter.
UBI1ZM 5UTR (PHI)	Maize *(Zea mays)*	5' untranslated region of the ubiquitin promoter.
UBI1ZM INTRON1 (PHI)	Maize *(Zea mays)*	Intron from the ubiquitin promoter.

SV40 NLS-V1	*Simian vacuolating virus 40*	Short peptide leader sequence that directs nuclear localization of the Cas9 protein in the cell.
CAS9 EXON1 (SP) (MO2)	*Streptococcus pyogenes*	Exon 1 of Cas9 endonuclease. Cas9 endonuclease introduces a double-strand break in the target endogenous DNA sequence.
ST-LS1 INTRON2	Potato *(Solanum tuberosum)*	Intron introduced for plant-optimized expression of Cas9 endonuclease.
CAS9 EXON2 (SP) (MO2)	*Streptococcus pyogenes*	Exon 2 of Cas9 endonuclease. Cas9 endonuclease introduces a double-strand break in the target endogenous DNA sequence.
PINII TERM	Potato *(Solanum tuberosum)*	Proteinase inhibitor II gene terminator to terminate transcription of the Cas9 gene.

Plasmid 5		
Genetic Element	**Origin**	**Function**
ATTL1	*Enterobacteria phage lambda*	Gateway™ cloning system recombination site to facilitate vector construction process.
ZM-U6 POLIII CHR8 PRO	Maize *(Zea mays)*	U6 polymerase III gene promoter to drive transcription of ZM-ERS1B and GUIDE RNA.
ZM-FEA3-CR3	Maize *(Zea mays)*	Sequence fragment complementary to the Cas9 endonuclease recognition site in the *FEA3-2* gene.
GUIDE RNA	*Streptococcus pyogenes*	Encodes crRNA-tracrRNA fusion transcript that binds and directs Cas9 endonuclease to the target site. ZM-FEA3-CR3 and GUIDE RNA together constitute chimeric guide RNA.
ZM-U6 POLIII CHR8 TERM	Maize *(Zea mays)*	U6 polymerase III gene terminator to terminate transcription of the 5’ chimeric guide RNA.
ATTL3	*Enterobacteria phage lambda*	Gateway™ cloning system recombination site to facilitate vector construction process.
KAN	*Escherichia coli*	Kanamycin resistance gene to facilitate identification of the plasmid-containing bacterial clones during the vector construction process.
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.

ds Oligo		
Genetic Element	**Origin**	**Function**

HDR-ZM-FEA3-2-5'	Maize *(Zea mays)*	5' (upstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.
ZM-FEA3-2 EXON2 (FRAG1) (ALT6)	Maize *(Zea mays)*	Part of the ZM-FEA3-2 coding sequence containing targeted base pair substitutions.
HDR-ZM-FEA3-2-3'	Maize *(Zea mays)*	3' (downstream) homology-directed recombination (HDR) arm to facilitate replacement the native cellular mechanism.

Description of scientific methodology that you used or intend to use to confirm that the intended genetic changes were achieved.

The intended base pair substitutions are confirmed by Next Generation Sequencing (NGS) analysis. Southern-by-sequencing (SbS) is used to confirm absence of unintentionally integrated DNA from the transformation plasmids in the final maize lines.

SbS is an efficient NGS-based sequencing tool described in detail in Zastrow-Hayes et al. (2015) and Brink et al. (2019). SbS analysis covers the sequences of the plasmids used in transformation and detects unique junctions that would be created between the plant genomic DNA and unintended sequences derived from the transformation plasmids, if unintended plasmid DNA integration has occurred. Only plants with no detected unintended plasmid-derived DNA are selected and advanced for further development.

As described above, CRISPR-Cas9 corn with improved grain yield stability is designed to not contain any genetic elements from the transformation plasmids and - more specifically - plant pest genetic material. Corn is not a plant pest and there is also no reason to believe that amino acid substitution in the *FEA3-2* gene would generate a plant pest, since no plant pest DNA is inserted into the plant genome, nor is yield phenotype associated with or likely to result in plant pest risk. Thus, we understand that APHIS would not consider CRISPR-Cas9 corn with improved drought tolerance and yield stability to be a regulated article under Part 340.

Additionally, the amino acid substitutions in the *FEA3-2* gene could have been achieved using conventional breeding techniques. For example, the gene-edited maize *fea-3-2* mutant allele described herein has been already obtained through EMS mutagenesis. SNPs are the most prevalent type of mutations in plants and the most common source of genetic variation between alleles of the species' gene pool (Glenn et al. 2017; Morgil et al., 2020). Furthermore, allelic differences between different varieties of the same species are often not limited by a single SNP or a single amino acid substitution (Tenaillon et al., 2001; Ching et al., 2002; Gore et al., 2009; Sun et al., 2018). Plant genomes are inherently variable and conventional breeding has been utilizing both spontaneous and induced genetic variability to develop new varieties (Mba, 2013; Glenn et al., 2017; Sun et al., 2018; Custers et al., 2019; Graham et al., 2020).

Therefore, coupled with the fact that the final gene-edited maize lines do not contain unintentionally integrated DNA from the transformation plasmids, these maize lines are indistinguishable from maize plants that could have resulted from native genome variability or be developed in a conventional breeding program.

We appreciate your review of this inquiry and are ready to address any questions you may have.

Sincerely,

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