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CORTEVA™ agriscience

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 Enhanced Yield Potential.

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) or targeted small insertion in the maize *zmm28* gene promoter resulting in plants with increased gene expression which is expected to enhance yield potential. As described below, we do not consider the final plant lines to be "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, base pair substitutions (edits) or targeted small inserts in the *zmm28* 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 *zmm28* gene encodes a MADS-box transcription factor which enhanced expression has been shown to result in plants with increased yield potential (Wu et al., 2019). A transgenic[2] overexpression approach has been previously utilized by Corteva by adding a copy of the *zmm28* coding sequence under the control of a moderate constitutive promoter to

[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.

[2] Due to inclusion of maize-optimized phosphinothricin acetyltransferase (*mo-pat*) into the construct

increase and extend the native *zmm28* gene expression (Wu et al., 2019). Here, the targeted base pair substitutions (edits) and the targeted small insertion were designed to enhance the expression of the native *zmm28* gene by introducing specific changes to its promoter region – namely, by creating an Expression Modulating Element (EME), which is a short sequence found in the maize genome.

In the first approach, we introduced **[** **]** within the *zmm28* gene promoter. This caused an almost 3-fold increase of the *zmm28* RNA expression in the preliminary assays. In the second approach, we inserted **[** **]** into the *zmm28* gene promoter, which is **[** **]**. Similarly, the preliminary assays showed a several-fold increase of the *zmm28* gene expression compared to the *zmm28* promoter without an EME. CBI Deleted CBI Deleted CBI Deleted CBI Deleted CBI Deleted

CRISPR-Cas9 gene editing was used to introduce targeted **[** **]** edits or targeted **[** **]** insertion using the "gene edit" (SDN2) technique (Podevin et al., 2013). The DNA repair template consisted of the sequence homologous to the targeted sequence near the double strand break (DSB) except for the specific **[** **]** edits or the **[** **]** insertion, respectively, and flanked on each side by homology arms (sequences identical to the sequences immediately upstream and downstream of the genomic sequence to be edited). Additionally, with the **[** **]** edit approach, an extra single base pair substitution was introduced into the DNA repair template. 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[3]. Homology arms induced the homology-directed recombination (HDR) DNA repair and resulted in repairing the DSB in a desired manner. CBI Deleted CBI Deleted CBI Deleted

Additional Information on the Edited Lines:

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

Description of intended phenotype(s)
Plants with enhanced yield potential.

Description of intended activity (movement or release)
Field trials to evaluate the *zmm28* gene expression and grain yield in field conditions.

Description of intended genetic change in final product
Targeted base pair substitutions (maize line 1) or targeted small base pair insertion (maize line 2)

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

Description of vector or vector agent
Biolistic transformation.

Each maize line was created by the particle bombardment with four plasmids containing the components of the CRISPR-Cas9 system and a double stranded (ds) oligonucleotide serving as the DNA repair template.

Maize line 1 (**[ ]** edit): Plasmids 1 -4 and ds Oligo1. **CBI Deleted**
Maize line 2 (**[ ]** insertion): Plasmids 1 -4 and ds Oligo2. **CBI Deleted**

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.
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 2		
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 3		
Genetic Element	**Origin**	**Function**
ATTB4	*Escherichia coli*	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	*Simian vacuolating virus 40*	Short peptide leader sequence that directs nuclear localization of the Cas9 protein in the cell.
CAS9 EXON1 (SP) (MO)	*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) (MO)	*Streptococcus pyogenes*	Exon 2 of Cas9 endonuclease. Cas9 endonuclease introduces a double-strand break in the target endogenous DNA sequence.
VIRD2 NLS	*Agrobacterium tumefaciens*	C-terminal bipartite Nuclear Localization Signal from the VirD2 endonuclease, which is intended to target proteins destined for transport into the nucleus.

PINII TERM	Potato *(Solanum tuberosum)*	Proteinase inhibitor II gene terminator to terminate transcription of the Cas9 gene.
ATTB1	*Escherichia coli*	Gateway™ cloning system recombination site to facilitate vector construction process.
MINI-ALLSTOPS6	synthetic	Synthetic sequence of stop codons designed to stop downstream translation of open reading frames.
CAMV35S ENH	Cauliflower mosaic virus	Upstream enhancer from Cauliflower mosaic virus.
LTP2 PRO	Barley *(Hordeum vulgare)*	Promoter from Ltp2 gene. This gene is expressed in the aleurone layer of kernels during the early to mid-phases of kernel development.
AM-CYAN1	*Anemonia majano*	Cyan fluorescent protein used as a visual marker.
CAMV35S TERM	Cauliflower mosaic virus	Terminator region from Cauliflower mosaic virus.
PSB1	synthetic	A synthetic sequence designed to facilitate PCR analysis of recombined FRT sites.
MINI-ALLSTOPS	synthetic	Synthetic sequence of stop codons designed to stop downstream translation of open reading frames.
MINI-ALLSTOPS4	synthetic	Synthetic sequence of stop codons designed to stop downstream translation of open reading frames.
MINI-ALLSTOPS3	synthetic	Synthetic sequence of stop codons designed to stop downstream translation of open reading frames.
ATTB2	*Escherichia coli*	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.
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.
ATTB3	*Escherichia coli*	Gateway™ cloning system recombination site to facilitate vector construction process.
SPC (VER2)	*Escherichia coli*	Spectinomycin resistance gene used as a selectable marker 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**
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 *zmm28* gene and GUIDE RNA.
ZM-ZMM28-CR1	Maize *(Zea mays)*	Sequence fragment complementary to the Cas9 endonuclease recognition site in the *zmm28* gene.
GUIDE RNA	*Streptococcus pyogenes*	Encodes crRNA-tracrRNA fusion transcript that binds and directs Cas9 endonuclease to the target site. ZM-ZMM28-CR1 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 1		
Genetic Element	**Origin**	**Function**
HDR-ZM-ZMM28-5'	Maize *(Zea mays)*	5’ (upstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.
ZM-ZMM28 PRO (ED85E)-FRAG2 (MOD1)	Maize *(Zea mays)*	Part of the *zmm28* promoter coding sequence containing targeted base pair substitutions.
HDR-ZM-ZMM28-3'	Maize *(Zea mays)*	3’ (downstream) homology-directed recombination (HDR) arm to facilitate replacement the native cellular mechanism.

ds Oligo 2		
Genetic Element	**Origin**	**Function**
HDR-ZM-ZMM28-5'	Maize *(Zea mays)*	5' (upstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.
[ ]	Maize *(Zea mays)*	**[ ]**
HDR-ZM-ZMM28-3'	Maize *(Zea mays)*	3' (downstream) homology-directed recombination (HDR) arm to facilitate replacement the native cellular mechanism.

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Description of scientific methodology that you used or intend to use to confirm that the intended genetic changes were achieved.
The targeted changes are confirmed in both maize lines 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 all 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 enhanced yield potential 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 targeted substitutions of several base pairs or targeted insertion of a short maize-derived DNA sequence in the *zmm28* promoter would generate a plant pest, since no plant pest DNA is inserted into the plant genome, nor is enhanced yield phenotype associated with or likely to result in plant pest risk. Thus, we understand that APHIS would not consider CRISPR-Cas9 corn designed to improve drought tolerance and yield stability as described herein to be a regulated article under Part 340.

Additionally, the base pair substitutions (edits) or small insertion in the maize *zmm28* gene promoter could have been achieved using conventional breeding. For example, the described substitution of **[ ]** within the *zmm28* gene promoter results in the **[ ]** DNA sequence found in genome of multiple maize varieties. Single Nucleotide Polymorphism (SNP) is the most prevalent type of mutations in plants and the most common source of genetic variation

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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 (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, is no reason to believe that similar mutations in the *zmm28* gene promoter would not be possible to occur spontaneously or through conventional mutagenesis. The short (**[ ]**) EME element introduced into the *zmm28* promoter also represents DNA sequence found in the maize genome. Indels of 20-1000 bp are yet another common type of the structural variation in plants and indels occurring in promoter regions are likely involved in regulation of gene expression (Zhang et al., 2008; Yang 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.

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

Sincerely,

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