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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 Improved Drought Tolerance and Yield Stability.

Dear Ms. Juarez,

Corteva Agriscience respectfully requests confirmation from the USDA-APHIS's Biotechnology Regulatory Services (BRS) regarding the regulatory status of four maize lines developed using CRISPR-Cas9 gene editing technology to introduce targeted base pair substitutions (edits) in the **[ ]** and **[ ]** genes and that are designed to result in plants with improved drought tolerance and yield stability. As described below, we do not consider these 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, the amino acid substitutions (edits) in the **[ ]** and **[ ]** genes 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.

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The maize **[ ]** and **[ ]** genes encode **[**

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[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.

] By introducing the same amino acid substitutions into the protein coding sequences of the **[ ]** and **[ ]** maize genes through gene editing we expect to develop plants with improved drought tolerance and yield stability. **CBI Deleted**

CRISPR-Cas9 gene editing was used to introduce several targeted base pair substitutions (edits) that lead to the corresponding amino acid substitutions using the "gene edit" (SDN2) technique (Podevin et al., 2013). Four maize lines were created where each line contains one amino acid substitution of interest. The DNA repair template consisted of the sequence homologous to the targeted sequence near the double strand break (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). Homology arms induced the homology-directed recombination (HDR) DNA repair and resulted in the desired repair of the DSB.

The summary of the targeted base pair and resulting amino acid substitutions (edits) is provided in the table below. Each **[ ]** maize line contains one amino acid substitution caused by one base pair substitution. Each **[ ]** maize line also contains a single targeted amino acid substitution however, it could have been done only by changing 2 base pairs. Additionally, for each maize line, 1 or 3 additional 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 not causing an amino acid substitution. **CBI Deleted** **CBI Deleted**

Gene	Targeted aa substitution*	Number of bp substitutions required to achieve the targeted aa substitution**	Number of additional bp substitutions (silent mutation) to protect the DNA repair template	Total number of targeted bp substitutions resulting in one aa substitution	
[ ]	[ ]	[ ]	3	4	CBI Deleted
[ ]	[ ]	[ ]	3	4	CBI Deleted
[ ]	[ ]	[ ]	1	3	CBI Deleted
[ ]	[ ]	[ ]	1	3	CBI Deleted

* aa – amino acid. Number indicates the position of the amino acid targeted for substitution.

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

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**bp – base pair. A – adenine, C – cytosine, G – guanine, T – thymine.

Additional Information on the Edited Lines:

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

Description of intended phenotype(s)
Plants with improved drought tolerance and yield stability.

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

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.

Maize lines **[ ]** and **[ ]** were each created by particle bombardment with Cas9-gRNA ribonucleoprotein (RNP) complex, 3 plasmids containing the helper components of the CRISPR-Cas9 system (selectable marker and two genes to improve regeneration frequencies), and a double stranded (ds) oligonucleotide serving as the DNA repair template. CBI Deleted

Maize lines **[ ]** and **[ ]** were each created by particle bombardment with 4 plasmids containing all components of the CRISPR-Cas9 system and a double stranded (ds) oligonucleotide serving as the DNA repair template. CBI Deleted

Description of constructs
Maize line **[ ]**: Plasmid 1, Plasmid 2, Plasmid 3, and ds Oligo 1. CBI Deleted
Maize line **[ ]**: Plasmid 1, Plasmid 2, Plasmid 3, and ds Oligo 2. CBI Deleted
Maize line **[ ]**: Plasmid 2, Plasmid 4, Plasmid 5, Plasmid 6, and ds Oligo 3 CBI Deleted
Maize line **[ ]**: Plasmid 2, Plasmid 4, Plasmid 5, Plasmid 6, and ds Oligo 4 CBI Deleted

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**
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.
LTP2 PRO	Barley *(Hordeum vulgare)*	Promoter of the *Ltp2* gene expressed in the aleurone layer of kernels during the early to mid-phases of development.

AM-CYAN1	*Anemonia majano*	Cyan fluorescent protein used as a visual marker.
CAMV35S TERM	Cauliflower mosaic virus	Terminator region.
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 5		
Genetic Element	**Origin**	**Function**
ATTL4	*Escherichia coli*	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-WUS2 (ALT1) coding sequence.
ZM-PLTP 5' UTR	Maize (*Zea mays*)	Phospholipid transfer protein gene 5' untranslated region for optimized expression of the 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 6			
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 **[ ]** and GUIDE RNA.	**CBI Deleted**
[ ]	Maize *(Zea mays)*	Sequence fragment complementary to the Cas9 endonuclease recognition site in **[ ]**gene.	**CBI Deleted**
GUIDE RNA	*Streptococcus pyogenes*	Encodes crRNA-tracrRNA fusion transcript that binds to Cas9 endonuclease and directs it to the target site. **[ ]** and GUIDE RNA together constitute chimeric guide RNA.	**CBI Deleted**
ZM-U6 POLIII CHR8 TERM	Maize *(Zea mays)*	U6 polymerase III gene terminator to terminate transcription of **[ ]** and GUIDE RNA.	**CBI Deleted**
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 Oligo1			
Genetic Element	**Origin**	**Function**	
[ ]	Maize *(Zea mays)*	3’ (downstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.	**CBI Deleted**
[ ]	Maize *(Zea mays)*	Part of the **[ ]** coding sequence containing targeted base pair substitutions.	**CBI Deleted**
[ ]	Maize *(Zea mays)*	5’ (upstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.	**CBI Deleted**

ds Oligo2		
Genetic Element	**Origin**	**Function**

[ ]	*Maize (Zea mays)*	3' (downstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.	**CBI Deleted**
[ ]	*Maize (Zea mays)*	Part of the **[ ]** coding sequence containing targeted base pair substitutions.	**CBI Deleted**
[ ]	*Maize (Zea mays)*	5' (upstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.	**CBI Deleted**

ds Oligo 3			
[ ]	Maize *(Zea mays)*	5' (upstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.	**CBI Deleted**
[ ]	Maize *(Zea mays)*	Part of the **[ ]** coding sequence containing targeted base pair substitutions.	**CBI Deleted**
[ ]	Maize *(Zea mays)*	3' (downstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.	**CBI Deleted**

ds Oligo 4			
[ ]	Maize *(Zea mays)*	5' (upstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.	**CBI Deleted**
[ ]	Maize *(Zea mays)*	Part of the **[ ]** coding sequence containing targeted base pair substitutions.	**CBI Deleted**
[ ]	Maize *(Zea mays)*	3' (downstream) homology-directed recombination (HDR) arm to facilitate replacement via the native cellular mechanism.	**CBI Deleted**

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 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 improved drought tolerance and 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 substitutions in the **[ ]** or **[ ]** genes would generate a plant pest, since no plant pest DNA is inserted into the plant genome, nor are drought tolerance or yield phenotypes 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 as described herein to be a regulated article under Part 340. **CBI Deleted**

Additionally, the amino acid substitutions in the **[ ]** and **[ ]** genes could have been achieved using conventional breeding techniques. As described above, Single Nucleotide Polymorphism (SNP) mutations resulting in the same amino acid substitutions have been generated in the **[ ]** and **[ ]** gene homologs in Arabidopsis through conventional 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. **CBI Deleted** **CBI Deleted**

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

Sincerely,

Annie Tang Gutsche, Ph.D.
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