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Dr. Michael J. Firko
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737

Re: Confirmation of Regulatory Status of CRISPR-Cas Corn with Improved Resistance to Northern Leaf Blight

Dear Dr. Firko:

DuPont Pioneer respectfully requests confirmation from the USDA-APHIS' Biotechnology Regulatory Services (BRS) regarding the regulatory status of corn (*Zea mays* L.) with improved resistance to Northern Leaf Blight (NLB) developed using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas gene editing technology. As described below, we do not consider the final plant line (further referenced herein as "CRISPR-Cas corn with improved resistance to NLB") to be a "regulated article" subject to APHIS oversight under 7 C.F.R. Part 340 with respect to introduction into and/or through the U.S. because it does 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 CRISPR-Cas corn with improved resistance to NLB is or will become a plant pest within the meaning of the Plant Protection Act. Indeed, the CRISPR-Cas corn with improved resistance to NLB resemble plants described in other inquiries that BRS has determined are not considered "regulated articles" under 7 C.F.R. Part 340 because they contain no introduced genetic material from a plant pest.

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

1. Description of the CRISPR-Cas gene editing technology and targeted allele replacement application

CRISPR-Cas gene editing is an *in vivo* DNA double-strand break (DSB) technology based on a bacterial endonuclease called Cas9. Cas9 endonuclease is guided to a targeted region of DNA by a short sequence part of which matches the sequence of the target DNA and introduces a double-strand break. DNA can be repaired in the absence or presence of a DNA repair template. In the presence of a DNA repair template, one application of the technology is that a region of sequence within the genome can be edited to match the repair template sequence, resulting in a desired characteristic. The DNA repair template contains the sequence of interest with incorporated edits 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 induce the native cellular mechanism referred to as homology-directed repair (HDR) and results in repairing the DSB in a desired manner. Such use of CRISPR-Cas gene editing technology is further referenced herein as the Gene-EDIT technology; the repair template DNA represents the edited version of the exact same gene and is used for allele replacement (Podevin *et al.*, 2013).

The components of the CRISPR-Cas Gene-EDIT technology are typically delivered into the plant cells by genetic transformation with plasmids carrying DNA coding sequences of these elements, including a DNA repair template. The CRISPR-Cas Gene-EDIT technology results in edits that can use, in this case, native maize sequence(s) of interest. If plasmid DNA sequence other than the DNA repair template sequence (i.e., unintended DNA sequence) is integrated into the plant genome, this material can be removed by genetic segregation through conventional breeding and confirmed as absent through subsequent molecular testing. The final plant line produced by the CRISPR-Cas Gene-EDIT technology is selected to contain the intended allele replacement and no unintended plasmid DNA sequence. It is therefore similar to plants that could result from native genome variability or be developed in a conventional breeding program.

2. Description of CRISPR-Cas Corn with Improved Resistance to NLB

Northern Leaf Blight (NLB) is a fungal disease caused by the hemibiotrophic fungal pathogen *Setosphaeria turcica* (anamorph *Exserohilum turcicum*, formerly known as *Helminthosporium turcicum*), and is of global economic importance. Corn grain loss has been reported in the range of 18% to 98% (Bair *et al.*, 1990; Gowda *et al.*, 1992; Perkins and Pedersen, 1987; Raymundo *et al.*, 1981; Sharma *et al.*, 2012). Resistance to specific races of the pathogen can be controlled by certain native disease resistance corn genes, such as *Ht1, Ht2, Ht3, Htm1, Htn1, HtN, HtP, ht4, and rt* (Hurni *et al.*, 2015; Ogliari *et al.*, 2005; Welz and Geiger, 2000). DuPont Pioneer scientists map-based cloned HtN/*Htn1* and validated two variations of a wall-associated kinase (WAK) gene, named NLB18, from the corn inbred lines PH26N and PH99N (Li and Wilson, 2011). The NLB18 gene is in the same genomic interval as HtN/*Htn1* (Simcox and Bennetzen, 1993).

WAK genes are widely found in corn and encode transmembrane proteins involved in pathogen recognition and cellular signaling (Zuo *et al.*, 2015). Pathogen recognition is a critical step leading to activation of a plant's innate immune system. The plant immune system consists of two interconnected tiers of receptors that govern recognition of pathogens. The first tier is the extracellular surface pattern recognition receptors (PRRs) that are activated by pathogen–associated molecular patterns (PAMPs), which trigger the PAMP-triggered immunity (PTI). The second tier is the intracellular nucleotide-binding leucine-rich repeat receptors that are activated by specific pathogen effectors, triggering the effector-triggered immunity. (Jones and Dangl, 2006) It is possible that NLB18 is part of a first-tier PRR response. Hurni *et al.* (2015) described an HtN/*Htn1* sequence in corn sharing 100% amino acid identity to PH99N/NLB18 and 90% identity to PH26N/NLB18, encoding a pattern recognition receptor kinase with an extracellular, wall-associated domain, supporting evidence that NLB18 is a first-tier response.

Introgressing a disease resistant allele into the desired germplasm is currently achieved through conventional breeding crosses. This requires many breeding cycles and in most cases is accompanied by genetic linkage drag. These limitations can be overcome by replacing the disease sensitive allele of the gene with the disease resistant allele of the same gene directly in elite inbred lines of interest using CRISPR-Cas Gene-EDIT technology.

DuPont Pioneer utilized a CRISPR-Cas Gene-EDIT approach to edit the NLB sensitive allele of the corn NLB18 gene with a resistance allelic version (designated in Table 1 as ZM-NLB18 (Res) allele) from the same locus, resulting in the improved disease resistant phenotype. The technical approach consisted of two objectives: (1) delete the NLB18 sensitive allele in a target genotype and (2) repair the targeted DSB with the NLB18 resistant allele from a disease resistant corn genotype. These two objectives were accomplished in a single step by supplying two guide RNAs to excise the NLB18 sensitive allele and a repair template DNA encoding the NLB18 resistant allele of that same gene. One guide RNA is homologous to a sequence in the NLB18 gene promoter region upstream of the putative transcription start site, and another guide RNA is homologous to the sequence in the 3'UTR (untranslated region) of the NLB18 gene. As described above, the native HDR mechanism facilitates gene editing using the repair template DNA with the respective genomic DNA sequences flanking the NLB18 sensitive allele. The resulting corn line contains the NLB resistant allele of the NLB18 gene at its native genomic location in place of the NLB sensitive allele of the NLB18 gene.

CRISPR-Cas corn with improved resistance to NLB was generated by biolistic transformation with seven plasmids simultaneously. A detailed list of the genetic elements, their origin, and function is presented in Table 1 below.

The intended change in CRISPR-Cas corn with improved resistance to NLB is the editing of the disease sensitive allele of the NLB18 gene with a disease resistant allele of the same gene. Next Generation Sequencing (NGS) technology is used to characterize the replacement of the disease sensitive allele with the disease resistant allele. Southern-by-Sequencing (SbS), which utilizes NGS technology, is currently used by DuPont Pioneer to confirm absence of unintended plasmid DNA sequence (Zastrow-Hayes *et al.*, 2015). SbS analysis covering the entire sequences of all plasmids is designed to detect unique junctions created between the plant genomic DNA and sequences derived from the transformation plasmids if plasmid DNA integration had occurred. Unintended integration of plasmid DNA sequences is then segregated by conventional breeding. Using this SbS analysis and breeding process, DuPont Pioneer selects and advances plants for commercial product development so that the final plant line contains the NLB18 disease resistance allele, but does not contain any unintended plasmid DNA sequence.

Table 1. List of genetic elements on the plasmids to generate CRISPR-Cas corn with improved resistance to NLB

Elements of the CRISPR-Cas Gene-EDIT technology are indicated by bold font

Genetic element	Origin	Function
Plasmid 1		
ATTL1-V1	Enterobacteria phage lambda	Modified Gateway™ (Thermo Fisher Scientific) cloning system recombination site to facilitate vector construction process.
Ubi-Promoter	Maize (*Zea mays* L.)	Polyubiquitin gene promoter; controls expression of the Cas9 coding sequence.
Ubi- 5UTR	Maize (*Zea mays* L.)	Polyubiquitin gene 5' untranslated region for optimized expression of the Cas9 coding sequence.
Ubi-Intron1	Maize (*Zea mays* L.)	Polyubiquitin gene intron 1 for optimized expression of the Cas9 coding sequence.
SV40 Nuclear localization signal	Simian vacuolating virus 40	Short peptide leader sequence that directs nuclear localization of the Cas9 protein in the cell.
CAS9 EXON1	*Streptococcus pyogenes*	Exon 1 of Cas9 endonuclease. Cas9 endonuclease introduces a double-strand break in the target endogenous DNA sequence.
ST-LS1 intron	Potato (*Solanum tuberosum* L.)	Intron introduced for plant-optimized expression of Cas9 endonuclease.
CAS9 EXON2	*Streptococcus pyogenes*	Exon 2 of Cas9 endonuclease. Cas9 endonuclease introduces a double-strand break in the target endogenous DNA sequence.
PINII terminator	Potato (*Solanum tuberosum* L.)	Proteinase inhibitor II gene terminator to terminate transcription of the Cas9 coding sequence.
ATTL2-V1	Enterobacteria phage lambda	Modified 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.[1]
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.
Plasmid 2		
ATTL1	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
U6 PolIII promoter	Maize (*Zea mays* L.)	U6 polymerase III gene promoter to drive transcription of ZM-NLB18-5' and GUIDE RNA.
ZM-NLB18-5'	Maize (*Zea mays* L.)	Encodes the transcript complementary to the sequence in the promoter region of the NLB18 sensitive allele to direct Cas9 endonuclease to the site for cleavage.
GUIDE RNA	*Streptococcus pyogenes*	Encodes crRNA-tracrRNA fusion transcript that directs Cas9 endonuclease to the target site. ZM-NLB18-5' and GUIDE RNA together constitute chimeric guide RNA.
U6 PolIII terminator	Maize (*Zea mays* L.)	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.[1]
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.
Plasmid 3		
ATTL1	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
U6 PolIII promoter	Maize (*Zea mays* L.)	U6 polymerase III gene promoter to drive transcription of ZM-NLB18-3' and GUIDE RNA.
ZM-NLB18-3'	Maize (*Zea mays* L.)	Encodes the transcript complementary to the sequence in the 3' UTR region of the NLB18 sensitive allele to direct Cas9 endonuclease to the site for cleavage.
GUIDE RNA	*Streptococcus pyogenes*	Encodes crRNA-tracrRNA fusion transcript that directs Cas9 endonuclease to the target site. ZM-NLB18-3' and GUIDE RNA together constitute chimeric guide RNA.
U6 PolIII terminator	Maize (*Zea mays* L.)	U6 polymerase III gene terminator to terminate transcription of the 3' 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.[1]
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.
Plasmid 4		
ATTL1	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
Ubi-Promoter	Maize (*Zea mays* L.)	Polyubiquitin gene promoter; controls expression of the NPTII coding sequence.
Ubi- 5UTR	Maize (*Zea mays* L.)	Polyubiquitin gene 5' untranslated region for optimized expression of the NPTII coding sequence.
Ubi-Intron1	Maize (*Zea mays* L.)	Polyubiquitin gene intron 1 for optimized expression of the NPTII coding sequence.
NPTII	*Escherichia coli*	Neomycin phosphotransferase gene conferring resistance to aminoglycoside antibiotics. Used as selectable marker in plant transformation.
PINII TERM	Potato (*Solanum tuberosum* L.)	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.[1]
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.
Plasmid 5		
ATTL1	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
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ZM-ODP2	Maize (*Zea mays* L.)	Coding sequence of the ovule development protein 2 gene to improve transformation frequencies.
OS-T28 TERM	Rice (*Oryza sativa* L.)	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.[1]
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.
Plasmid 6		
ATTL4	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
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ZM-WUS2	Maize (*Zea mays* L.)	Coding sequence of the wuschel 2 gene to improve transformation frequencies.
ZM-IN2-1 TERM	Maize (*Zea mays* L.)	*In2-1* gene terminator to terminate transcription of the ZM-WUS2 coding sequence.
AT-5-IV-2 INS	*Arabidopsis thaliana* L.	Putative insulator sequence to limit ZM-WUS2 transcript read-through.
ATTR1	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.[1]
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.
Plasmid 7		
ATTL4	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.
HDR-NLB18-5'	Maize (*Zea mays* L.)	5' (upstream) homology-directed repair (HDR) arm to facilitate replacement of the NLB18 sensitive allele with the NLB18 resistant allele (designated as ZM-NLB18 (Res) allele) via the native cellular mechanism.
ZM-NLB18 (Res) PRO	Maize (*Zea mays* L.)	ZM-NLB18 (Res) promoter to control expression of ZM-NLB18 (Res) coding sequence.
ZM-NLB18 (Res) EXON1	Maize (*Zea mays* L.)	Exon 1 (part of the coding sequence) of the ZM-NLB18 (Res) allele.
ZM-NLB18 (Res) INTRON1	Maize (*Zea mays* L.)	Intron 1 (part of the non-coding sequence) of the ZM-NLB18 (Res) allele.
ZM-NLB18 (Res) EXON2	Maize (*Zea mays* L.)	Exon 2 (part of the coding sequence) of the ZM-NLB18 (Res) allele.
ZM-NLB18 (Res) INTRON2	Maize (*Zea mays* L.)	Intron 2 (part of the non-coding sequence) of the ZM-NLB18 (Res) allele.

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ZM-NLB18 (Res) EXON3	Maize (*Zea mays* L.)	Exon 3 (part of the coding sequence) of the ZM-NLB18 (Res) allele.
ZM-NLB18 (Res) TERM	Maize (*Zea mays* L.)	ZM-NLB18 (Res) terminator to terminate transcription of the coding sequence of the ZM-NLB18 (Res) allele.
HDR-NLB18-3'	Maize (*Zea mays* L.)	3' (downstream) homology-directed repair (HDR) arm to facilitate replacement of the ZM-NLB18 sensitive allele with the ZM-NLB18 (Res) allele via the native cellular mechanism.
ATTR1	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.[1]
PUC ORI	*Escherichia coli*	Origin of replication to facilitate plasmid propagation in bacterial cells.
SC-CEN6 ARS	*Saccharomyces cerevisiae*	Origin of replication for *URA3* marker gene to facilitate NLB18 (Res) template vector assembly.
SC-URA3 PRO	*Saccharomyces cerevisiae*	Promotor for *URA3* marker gene to facilitate NLB18 (Res) template vector assembly.
SC-URA3	*Saccharomyces cerevisiae*	*URA3* marker gene to facilitate NLB18 (Res) template vector assembly.
SC-URA3 TERM (TR1)	*Saccharomyces cerevisiae*	Terminator for *URA3* marker gene to facilitate NLB18 (Res) template vector assembly.
ATTL4	Enterobacteria phage lambda	Gateway™ cloning system recombination site to facilitate vector construction process.

[1] The KAN gene is used as a bacterial selectable marker for plasmid maintenance only. As such, there is no need for a eukaryotic promoter.

3. APHIS Jurisdiction

APHIS' regulations for genetically engineered organisms are codified at 7 CFR Part 340 ("Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There is Reason To Believe Are Plant Pests"). Under the provisions of these regulations at Section 340.1, an organism is deemed to be a regulated article subject to APHIS oversight if that organism has been "altered or produced through genetic engineering," but only under circumstances in which an associated donor organism, recipient organism, or vector or vector agent used to produce the article is either:

(i) listed in Section 340.2 and meets the definition of "plant pest," or

(ii) an unclassified organisms and/or an organism whose classification is unknown (or the Administrator otherwise determines that the organism is a plant pest or has reason to believe it is a plant pest).

Further, in contrast to plants that are regulated under Part 340 because they (a) are genetically engineered with the use of plant pest donor organisms, recipient organisms, or vectors or vector agents, and (b) contain the inserted plant pest genetic material, APHIS has clarified that subsequently bred null segregant lines created from the regulated

parent plants will not themselves be considered regulated articles under Part 340 as long as those lines contain no inserted genetic material from a plant pest and APHIS has no reason to believe they are plant pests[1].

As described above, the CRISPR-Cas corn with improved resistance to NLB does not contain any genetic elements from the transformation plasmids and - more specifically - plant pest genetic material. There is also no reason to believe that changes to the plant genome generated by the NLB18 gene replacement would generate a plant pest, since no plant pest DNA is inserted into the plant genome during the process. Thus, considering the provisions of 7 CFR Part 340 and the subsequent APHIS guidance, we understand that APHIS would not consider CRISPR-Cas corn with improved resistance to NLB to be a regulated article under Part 340.

4. Conclusion

In summary, CRISPR-Cas corn with improved resistance to NLB generated using the CRISPR-Cas Gene-EDIT technology is not a plant pest, does not incorporate any plant pest material, and there is no basis to believe that plants of CRISPR-Cas corn with improved resistance to NLB are plant pests within the meaning of the Plant Protection Act. For all of the reasons provided above, we respectfully ask for APHIS' confirmation that the CRISPR-Cas corn with improved resistance to NLB described above is not a regulated article subject to APHIS oversight under 7 C.F.R. Part 340.

We thank APHIS in advance for your consideration of this request. If you have any questions, we would welcome the opportunity to meet with you at a convenient time to further discuss CRISPR-Cas corn with improved resistance to NLB.

Sincerely,

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[1] APHIS responses to: Mr. Richael and Ms. Rood (Dec. 2, 2016); Mr. W. Haun (Sep. 15, 2016); Dr. D.H. Schmidt (Apr. 18, 2016); Dr. Yinong Yang (Apr. 13, 2016); Mr. W. Haun (Feb. 11, 2016); Dr. Raab, Agrivida (Nov. 12, 2015); Mr. Cullen, Arnold & Porter (Nov. 12, 2015); Dr. Yang, Iowa State University (May 22, 2015); Dr. Mathus, Cellectis Plant Sciences (May 20, 2015, May 5, 2015, and Aug. 28, 2014); Dr. Mackenzie, University of Nebraska (Jun. 6, 2012); APHIS Response to Dr. Lewis, North Carolina State University (Oct. 27, 2011).

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