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*By APHIS BRS Document Control Officer at 3:39 pm, Dec 18, 2015*

December 14, 2015

Michael Firko, Ph.D.
Deputy Administrator, Biotechnology Regulatory Services
USDA-APHIS
4700 River Road, Unit 98
Riverdale, MD 20737

Re: Confirmation of Regulatory Status of Waxy Corn Developed by CRISPR-Cas Technology

Dear Dr. Firko:

DuPont Pioneer respectfully requests confirmation from the USDA-APHIS's Biotechnology Regulatory Services (BRS) regarding the regulatory status of waxy corn developed using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas genome editing technology. As described below, we do not consider the final plant line (further referenced herein as "CRISPR-Cas waxy corn") to be a "regulated article" subject to APHIS oversight under 7 C.F.R. Part 340 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 waxy corn is or will become a plant pest within the meaning of the Plant Protection Act. Indeed, the CRISPR-Cas waxy corn plants 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 genome editing technology and gene knockout application

CRISPR-Cas genome editing is an *in vivo* DNA double-strand break (DSB) technology based on a bacterial endonuclease called Cas9. It utilizes a combination of protein-DNA and RNA-DNA pairing to direct targeted double strand breaks in the DNA sequence of interest. 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 DSB. In one of the applications of CRISPR-Cas genome editing technology, the generated DSB is subsequently repaired by a native cellular mechanism called "non-homologous end joining". This process can lead to deletion or addition

of base pairs at the repair site which, in turn, often results in inactivation of a target gene. Such use of CRISPR-Cas genome editing technology is further referenced herein as the "gene knockout" technology.

The components of the CRISPR-Cas gene knockout technology are typically delivered into the plant cells by genetic transformation with plasmids carrying DNA coding sequences of these elements. Notwithstanding, the CRISPR-Cas gene knockout technology can function without inserting any plasmid components into the plant genome. If plasmid DNA sequence is randomly integrated into the plant genome, this material can be removed by genetic segregation through conventional breeding processes and confirmed through subsequent molecular testing (generation of a null segregant line). The final plant line produced by the CRISPR-Cas gene knockout technology is selected to contain no randomly integrated DNA material and is indistinguishable from plants that could result from native genome variability or be developed in a conventional breeding program.

## 2. Description of CRISPR-Cas Waxy Corn

Corn starch accounts for over 70% of the total kernel weight (Whitt et al (2002) PNAS USA 99:12595-12962) and is composed of two polysaccharides - amylose and amylopectin, with an approximate ratio of 27% to 73% (respectively) in normal dent corn[1]. "Waxy corn" is a specialty corn with the starch composed exclusively of amylopectin; such cornstarch is a valuable commodity due to its superior physico-chemical properties and is widely used in the food and paper industry. The waxy corn phenotype can be achieved by inactivation of the endogenous waxy gene (*Wx1*) that encodes a granule-bound starch synthase catalyzing production of amylose.

DuPont Pioneer utilized a CRISPR-Cas gene knockout approach to inactivate the corn *Wx1* gene resulting in the waxy corn phenotype. This was accomplished by introduction of two guide RNAs to generate two DSBs. One guide RNA is homologous [ ], and another guide RNA is homologous [ ]. Through the CRISPR-Cas gene knockout process described above, the result was a corn line with the deletion of the DNA sequence between the two DSB sites, causing inactivation of the *Wx1* gene. **CBI** **CBI**

CRISPR-Cas waxy corn was generated by biolistic transformation with six plasmids. A detailed list of the genetic elements, their origin, and function is presented in the table below.

Unintended integration of DNA sequences from the six plasmids is analyzed using a comprehensive molecular analysis and segregated by conventional breeding if needed. The final plant line, CRISPR-Cas waxy corn, is a null segregant line that contains the targeted deletion of the *Wx1* gene sequence but does not contain any inserted DNA from the six plasmids.

[1] http://corn.agronomy.wisc.edu/Management/pdfs/NCH10.pdf

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**Table. List of genetic elements on the plasmids to generate CRISPR-Cas waxy corn**
Elements of the CRISPR-Cas gene knockout 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*) | Polyubiquitin gene promoter; controls expression of the Cas9 coding sequence. |
| **Ubi- 5UTR** | Maize (*Zea mays*) | Polyubiquitin gene 5' untranslated region for optimized expression of the Cas9 coding sequence. |
| **Ubi-Intron1** | Maize (*Zea mays*) | 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*) | 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*) | 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. |
| 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*) | U6 polymerase III gene promoter to drive transcription of ZM-WXY-5' and GUIDE RNA. |
| **ZM-WXY-5'** | Maize (*Zea mays*) | Encodes the transcript [ ] to direct Cas9 endonuclease to the site for cleavage. CBI |
| **GUIDE RNA** | *Streptococcus pyogenes* | Encodes crRNA-tracrRNA fusion transcript that directs Cas9 endonuclease to the target site. ZM-WXY-5' and GUIDE RNA together constitute chimeric guide RNA. |
| **U6 PolIII terminator** | 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. |

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| | | |
|---|---|---|
| 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** | | |
| ATTL1 | Enterobacteria phage lambda | Gateway™ cloning system recombination site to facilitate vector construction process. |
| **U6 PolIII promoter** | Maize (*Zea mays*) | U6 polymerase III gene promoter to drive transcription of ZM-WXY-3' and GUIDE RNA. |
| **ZM-WXY-3'** | Maize (*Zea mays*) | Encodes the transcript [ ] to direct Cas9 endonuclease to the site for cleavage. **CBI** |
| **GUIDE RNA** | *Streptococcus pyogenes* | Encodes crRNA-tracrRNA fusion transcript that directs Cas9 endonuclease to the target site. ZM-WXY-3' and GUIDE RNA together constitute chimeric guide RNA. |
| **U6 PolIII terminator** | Maize (*Zea mays*) | 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. |
| 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*) | Polyubiquitin gene promoter; controls expression of the NPTII coding sequence. |
| **Ubi- 5UTR** | Maize (*Zea mays*) | Polyubiquitin gene 5' untranslated region for optimized expression of the NPTII coding sequence. |
| **Ubi-Intron1** | Maize (*Zea mays*) | Polyubiquitin gene intron1 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*) | 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 5** | | |
| ATTL1 | Enterobacteria phage lambda | Gateway™ cloning system recombination site to facilitate vector construction process. |
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| **ZM-ODP2** | Maize (*Zea mays*) | Coding sequence of the ovule development protein 2 gene to improve transformation 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 6** | | | |
| ATTL4 | Enterobacteria phage lambda | Gateway™ cloning system recombination site to facilitate vector construction process. | |
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| **ZM-WUS2** | Maize (*Zea mays*) | Coding sequence of the wuschel 2 gene to improve transformation frequencies. | |
| **ZM-IN2-1 TERM** | Maize (*Zea mays*) | *In2-1* gene terminator to terminate transcription of the ZM-WUS2 coding sequence. | |
| AT-5-IV-2 INS | *Arabidopsis thaliana* | 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. | |
| PUC ORI | *Escherichia coli* | Origin of replication to facilitate plasmid propagation in bacterial cells. | |

## 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 are plant pests[2].

As described above, the CRISPR-Cas waxy corn produced using CRISPR-Cas gene knockout technology to inactivate the *Wx1* gene 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 *Wx1* gene inactivation 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 waxy corn to be a regulated article under Part 340.

## 4. Conclusion

In summary, CRISPR-Cas waxy corn generated using the CRISPR-Cas gene knockout technology is not a plant pest, does not incorporate any plant pest material, and there is no basis to believe that CRISPR-Cas waxy corn plants 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 waxy corn 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 waxy corn.

Sincerely,

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[2] APHIS responses to: 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).