Biotechnology Notification File No. 000171
Center for Veterinary Medicine Note to the File

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To: Administrative Record, BNF No. 000171

Subject: Event DP202216 Corn

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Purpose

This document summarizes the Food and Drug Administration (FDA) Center for Veterinary Medicine's (CVM, we) evaluation of biotechnology notification file (BNF) number 000171. Pioneer Hi-Bred International, Inc. (Pioneer) submitted a safety and nutritional assessment for a genetically engineered (GE) corn, transformation event DP-202216-6 (hereafter referred to as DP202216 corn) and additional information afterwards. CVM evaluated the information in Pioneer's submissions to ensure that regulatory and safety issues regarding animal food derived from DP202216 corn have been resolved prior to commercial distribution. FDA's Center for Food Safety and Applied Nutrition summarizes its evaluation of uses of DP202216 corn in human food in a separate document.

In CVM's evaluation, we considered all of the information provided by Pioneer as well as publicly available information and information in the agency's files. Here we discuss the outcome of the consultation for animal food use, but do not intend to restate the information provided in the final consultation in its entirety.

Intended Effects

The first intended effect of the modification in DP202216 corn is to enhance grain yield potential from the new plant variety. To confer this trait, Pioneer introduced an additional copy of the native *zmm28* gene that encodes for the ZMM28 protein, a MADS-box transcription factor. The introduced gene leads to increased and extended expression of ZMM28. Pioneer also introduced the *pat* gene from *Streptomyces viridochromogenes*. This gene encodes phosphinothricin N-acetyltransferase (PAT), which confers tolerance to glufosinate ammonium herbicides.

Regulatory Considerations

The purposes of this evaluation are (1) to assess whether Pioneer has introduced into animal food a substance requiring premarket approval as a food additive and (2) to determine whether use of the new plant variety in animal food raises other regulatory issues with respect to the Federal Food, Drug, and Cosmetic Act (FD&C Act).

The Environmental Protection Agency (EPA) regulates herbicides under the FD&C Act and the Federal Insecticide, Fungicide, and Rodenticide Act. Under EPA regulations, the herbicide residues in DP202216 corn are considered pesticide residues.

Genetic Modification and Characterization

Introduced DNA and Transformation Method

Pioneer transformed immature corn embryos, which were obtained from inbred corn line, PH17AW, with plasmid PHP40099 using disarmed *Agrobacterium tumefaciens*-mediated transformation.[1] The transfer-DNA (T-DNA) region within the plasmid contained two expression cassettes between the left and right border sequences. The first cassette contained nucleotide sequences from the coding region of *zmm28* gene from *Zea mays* L., with regulatory elements, including the promoter region of *gos2* gene from *Z. mays* L., intron region of the *ubiquitin* gene 1 from *Z. mays* L., bacteriophage lambda integrase recombination site (*attB1*), bacteriophage lambda integrase recombination site (*attB2*), and terminator region of *proteinase inhibitor II* gene from *Solanum tuberosum*. Additional copies of bacteriophage lambda integrase recombination sites (*attB4* and *attB3*) and bacteriophage P1 recombination sites (*loxP*) were located upstream and downstream of the zmm28 expression cassette.[2] The second cassette included a codon-optimized *phosphinothricin N-acetyltransferase* (*pat*) gene, which encodes the same amino acid sequence as the PAT protein that is expressed in *S. viridochromogenes*. The upstream regulatory elements include the promoter region, 5´ untranslated region, and intron region of the *Z. mays ubiquitin* gene 1 and a flippase recombination target site (*FRT*), *FRT1*, from *Saccharomyces cerevisiae*, while downstream of the *pat* gene is the terminator region of *proteinase inhibitor II* gene from *S. tuberosum*, followed by *FRT87*, a modified *S. cerevisiae* site.

Following transformation, calli were grown in selection medium[3], plants were then regenerated and grown to maturity. Genomic DNA was isolated from the leaves of established plants and polymerase chain reaction (PCR) was used to confirm the presence of the T-DNA region of plasmid PHP40099. Additional breeding steps were conducted to generate homozygous and hemizygous plants used in the characterization of the genetic insertion, inheritance studies, and gene expression studies.

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[1] The transformation method was essentially as described by Zhao, Z.-Y., W. Gu, T. Cai, L. Tagliani, D. Hondred, D. Bond, S. Schroeder, M. Rudert, and D. Pierce. 2001. High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. Molecular Breeding 8:323-333.

[2] The firm states the recombination sites, *attB*, *loxP*, and *FRT*, do not result in a recombination event without a recombinase enzyme(s) that is not naturally present in plants.

[3] The selection media contained glufosinate ammonium herbicide for selection of transformants and carbenicillin for inhibition of *A. tumefaciens*.

Pioneer characterized the number of T-DNA inserts, organization of the insert that is present in DP202216 corn, and the absence of vector backbone sequences using Southern-by-Sequencing method.[4] The parental cultivar, PH17AW, was used as the comparator in these analyses. Nucleotide sequences that contained both corn genomic DNA and T-DNA sequences (junction sequences) were used to identify the number of copies of the T-DNA that was inserted into the genome of PH17AW. In addition, Pioneer harvested leaves from DP202216 corn plants, generated complementary DNA, and amplified DNA using primers specific for native and introduced *zmm28* genes. The PCR products were sequenced and bioinformatics tools were used to align these sequences to the *zmm28* gene[5] in the B73 maize reference genome. Pioneer reports that a single, intact copy of the T-DNA was inserted in a non-repetitive and non-coding region of the genome of DP202216 corn and there were no detectable rearrangements or truncations in the inserted DNA, with the exception of minor truncations of the right and left border regions.[6] Pioneer also reports that sequences from the backbone region of the vector are not present in DP202216 corn.

Pioneer evaluated the stability of the inserted T-DNA in DP202216 corn in two self-pollinated lines and in three out-crossed lines of DP202216 corn using Southern blot analysis. Pioneer digested genomic DNA with *Nco I* restriction enzyme, prepared the fragmented DNA for Southern blot analysis, and hybridized with labelled sequences for the *zmm28* and *pat* genes. Pioneer observed event-specific bands unique to DP202216 corn which had similar migration patterns across generations and bands that aligned with the native *zmm28* gene and homologous sequences that were present in control corn genomic DNA samples. Stability of the individual genes was also assessed by PCR. Pioneer assessed segregation of the *pat* gene using the herbicide tolerance phenotype in plants treated with glufosinate ammonium herbicide. Pioneer concludes the desired genotype segregated as a single loci according to expected Mendelian principles.

Pioneer performed bioinformatics analyses using the nucleotide sequences obtained for the T-DNA insert and the bordering genomic junction sequences to determine whether insertion of the introduced DNA created any potential open reading frames (ORFs) that could encode for putative polypeptides. Pioneer evaluated the putative polypeptides against a database that included proteins in the UniProtKB/Swiss-Prot database filtered for molecular function by keywords that could imply toxicity or adverse health effects. Based on the results of bioinformatics analyses, Pioneer concludes that the putative polypeptides did not align with sequences in its toxin database.

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[4] Southern-by-Sequencing technique utilizes probes that are homologous to the transformation plasmid to capture hybridizing genomic DNA. The captured genomic DNA is then sequenced using whole genome sequencing and the results were analyzed using bioinformatics tools.

[5] GenBank accession number NP_001105155.1.

[6] Wu, J., S.J. Lawit, B. Weers, J. Sun, N. Mongar, J. Van Hemert, R. Melo, X. Meng, M. Rupe, J. Clapp, K.H. Collet, L. Trecker, K. Roesler, L. Peddicord, J. Thomas, J. Hunt, W. Zhou, Z. Hou, M. Wimmer, J. Jantes, H. Mo, L. Liu, Y. Wang, C. Walker, O. Danilevskaya, R.H. Lafitte, J.R. Schussler, B. Shen, and J.E. Habben. 2019. Overexpression of zmm28 increases maize grain yield in the field. Proc. Nat. Acad. Sci. 116:23850-23858.

Protein Safety

Pioneer used a weight of evidence approach to demonstrate that the PAT protein and the increases in the ZMM28 protein do not raise safety concerns. The ZMM28 protein is described as a MIKC protein because it contains an N-terminal MADS-box[7] domain, which is involved in binding to DNA, followed by an Intervening region, a Keratin-like box, and a C-terminal domain that is necessary for activity and complex formation.[6] Pioneer verified using bioinformatics analysis that the amino acid sequence of the introduced ZMM28 protein has the same sequence and contains the same MIKC domain as the ZMM28 protein that naturally occurs in corn. Pioneer also notes that ZMM28 protein shares 94, 75, and 76% amino acid identity with MADS-box proteins in sorghum, barley, and rice.[6,8] Western blot analysis was used to confirm that the introduced ZMM28 protein in DP202216 corn and the ZMM28 protein from sweet corn varieties have the same molecular weight and immunoreactivity. Pioneer concludes that the introduced ZMM28 protein is equivalent to the ZMM28 protein that has a history of safe use in animal food.

The PAT protein from *S. viridochromogenes* is an acetyltransferase composed of 183 amino acids. PAT protein confers tolerance to the herbicide glufosinate ammonium by acetylating the active ingredient, L-phosphinothricin, inactivating it. Pioneer verified using bioinformatics analysis that the amino acid sequence resulting from the translation of the codon-optimized *pat* gene is identical to the amino acid sequence of the PAT protein that is expressed in *S. viridochromogenes*. Western blot analysis was used to confirm that the PAT protein in DP202216 corn had the same molecular weight and immunoreactivity as a microbially derived reference PAT protein and confirmed that the PAT protein was not detected in the near-isoline control corn. In addition, Pioneer conducted an *in silico* analysis using the amino acid sequence for the PAT protein to determine whether there were any potential polypeptides that align with sequences in its toxin database and concluded that the amino acid sequence for PAT protein did not align with any sequences in its database. Pioneer highlights that the safety of the PAT protein has been assessed previously.[9] Based on the weight of the evidence, Pioneer concludes that the PAT protein expressed in DP202216 corn is unlikely to raise safety concerns.

Expression Levels of Proteins in DP202216 Corn

Pioneer quantified the amounts of the two introduced proteins that are present in DP202216 corn. Tissue samples were collected for leaf (V6, V9, R1, R4, and R6 growth

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[7] MADS is an acronym derived from the four founding members of this transcription factor family: Minichromosomal maintenance1 from *Saccharomyces cerevisiae*, Agamous from *Arabidopsis thaliana*, Deficiens from *Antirrhinum majus*, and Serum response factor from humans. As described in Schilling, S., S. Pan, A. Kennedy, and R. Melzer. 2018. MADS-box genes and crop domestication: The jack of all traits. J. Exp. Bot. 69:1447-1469.

[8] Anderson, J.A., S. Brustkern, B. Cong, L. Deege, B. Delaney, B. Hong, S. Lawit, C. Mathesius, J. Schmidt, J. Wu, J. Zhang, and C. Zimmermann. 2019. Evaluation of the history of safe use of the maize ZMM28 protein. J. Agric. Food Chem. 67: 7466-7474.

[9] Hérouet, C., D.J. Esdaile, B.A. Mallyon, E. Debruyne, A. Schulz, T. Currier, K. Kendrickx, R.-J. van der Klis, and D. Rouan. 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the *pat* and *bar* sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regul. Toxicol. Pharmacol. 41:134-149.

stages), pollen (R1 growth stage), forage (R4 growth stage), whole plant growth stages (V9, R1, and R6 growth stages), and grain (R6 growth stage)[10] during the 2017 growing season at six sites in the United States and one site in Canada. Each site included DP202216 corn and a near-isoline control maize, which were planted in a randomized complete block design containing four blocks. The tissue samples were collected non-systematically and each tissue type was homogenized into powder prior to analysis. The amounts of ZMM28 and PAT proteins present in the samples were determined by enzyme linked immunosorbent assay (ELISA), with the exception of the ZMM28 protein in grain. A Western blot method was used to measure the concentration of ZMM28 protein in grain because the grain matrix interfered with the ELISA method. Pioneer states that the PAT protein is not expressed in the control line and, thus, PAT protein was not measured in the control tissue samples. Pioneer states that the endogenous ZMM28 protein is differentially expressed in the control; expression was slightly above the limit of detection in leaf samples obtained from plants at the V6 growth stage and was highest in samples obtained at the V9 to R1 growth stages (0.21 nanograms/milligram tissue DW). Pioneer states that the introduced ZMM28 protein was constitutively expressed in DP202216 corn, which resulted in extended and higher ZMM28 protein levels during V4-R4 growth stages when compared to the control (the highest levels, 0.28-0.32 nanograms/milligram tissue DW, being between V9 and R1 growth stages). Pioneer reports that the highest concentration of PAT protein, 88 nanograms of PAT protein/milligram dry weight in leaf tissue, was obtained at R4 growth stage. The concentration of PAT protein in whole plant material ranged from 32 to 21 nanograms/milligram DW at V9 to R6 growth stages, respectively. The concentration of PAT protein in grain at the R6 stage was 15 nanograms/milligram dry weight.

Pioneer notes that a weight of the evidence approach was used to demonstrate that the PAT protein expressed in DP202216 corn is identical to the PAT protein that was expressed in other new plant varieties (BNF 73 and 81) that have been safely grown and used in the U.S. Pioneer notes that the ZMM28 protein is a native corn protein and has a history of safe use. Based on the history of safe use and calculated livestock dietary exposure assessment, Pioneer concludes that the risk of adverse effects from ZMM28 and PAT proteins in DP202216 corn is low.

Animal Food Use

Corn (*Zea mays* L.) is a commodity crop grown worldwide for various uses, including human and animal food. Corn is an important crop for animal food. Corn grain and by-products of corn processing may be included in diets for most animal species. Corn silage is a readily digestible, high energy, fermented forage product. It is fed primarily to ruminants (e.g., cattle, sheep, and goats). For animal nutrition, corn is considered to be an important source of energy, essential fatty acids and some of the essential amino acids.

[10] Pioneer states that growth stages are defined in L.B. Abendroth, R.W. Elmore, M.J. Boyer, and S.K. Marlay. 2011. Corn growth and development. PMR 1009. Iowa State University of Science and Technology, Cooperative Extension Service, Ames, Iowa.

Composition

Scope of Analysis

Pioneer determined whether there were any unintended changes in the nutrient composition of grain and forage from DP202216 corn when compared to a non-GE, near-isogenic corn (control), and 16 reference corn hybrids (four hybrids planted at each location) that were grown and harvested under similar conditions. The selected components were based on the Organisation for Economic Cooperation and Development (OECD) corn composition consensus document.[11]

Study Design

Pioneer conducted field trials in 2017.[12] There were eight locations, with seven locations in the United States and one in Canada. The corn varieties were planted using a randomized complete block design with four replicate plots at each field location. One forage sample per plot was harvested at the R4 growth stage[13]; plants were chopped into sections (≤ 7.6 centimeters long). Grain samples, which consisted of grain pooled from five ears from each replicate at each location, were pooled prior to sub-sampling. Forage and grain samples were transported in chilled containers and then stored frozen until compositional analysis was performed.

Pioneer statistically compared each component for DP202216 corn and the control across locations using a linear mixed model analysis of variance. The false discovery rate adjustment was also used to control for false positive outcomes across all components analyzed using linear mixed models and adjusted *p*-values are also reported. For two components Fisher's exact test was used for the analysis. Components were expressed on a dry matter basis prior to statistical analysis and forage and grain moisture was not included in the statistical analyses. When a value for a component was less than the lower limit of quantitation (LLOQ) for the analytical method, a value equal to half the LLOQ was assigned to this sample. Differences between DP202216 corn and control with a $P \leq 0.05$ in the mixed model or Fisher's exact test were considered to be statistically different. Any observed differences in a component between DP202216 corn and control were compared with tolerance intervals derived from non-GE corn lines that were grown in the United States and Canada, and South America between 2003 and 2015 (described as 93 commercial corn lines and 88 unique environments). If the range of DP202216 corn contained individual values that fell outside the tolerance interval, then these values were compared to the range of values in the public literature and to the range of values obtained for the non-GE varieties that were concurrently grown at the eight locations.

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[11] OECD (2002) Consensus Document on Compositional Considerations for New Varieties of Maize (*Zea mays*): Key Food and Feed Nutrients, Anti-Nutrients and Secondary Plant Metabolites. Organisation for Economic Co-operation and Development, ENV/JM/MONO(2002)25. OECD, Paris.

[12] Anderson, J.A., B. Hong, E. Moellring, S. TeRonde, C. Walker, Y. Wang, and C. Maxwell. 2019. Composition of forage and grain from genetically modified DP202216 maize is equivalent to non-modified conventional maize (Zea mays L.). GM Crops Food 10:77-89.

[13] A total of 32 samples were obtained for analysis.

Results of Analyses – Forage

Pioneer reports values for proximates (crude protein, crude fat, carbohydrates by calculation, and ash), fiber (acid detergent fiber (ADF) and neutral detergent fiber (NDF)), calcium, and phosphorus. Pioneer found no statistically significant differences between the mean values for these components in forage from DP202216 corn and the control. In addition, the mean value for each component in DP202216 corn fell within the range of values for the tolerance interval.

Results of Analyses – Grain

Pioneer measured proximates, fiber (ADF, NDF, crude fiber, and total dietary fiber), 18 amino acids, nine minerals, 15 fatty acids (for six of the fatty acids more than 50% of the values fell below the LLOQ in both DP202216 corn and control), 11 vitamins plus total tocopherols (for vitamin B2, beta-tocopherol, and delta-tocopherol most or all of the values were below the LLOQ), four secondary metabolites (for furfural most of the values were below the LLOQ), and three anti-nutrients. Pioneer states that there were no statistically significant differences in any of the components with values greater than LLOQ, with the exception of thiamin, niacin, and three amino acids. Although the concentrations of these analytes were significantly different in grain obtained from DP202216 corn when compared to the control, the false discovery rate adjusted probability values were not statistically significant. Pioneer also states that the mean values fell within the range of values for the tolerance interval. Pioneer concludes that the differences in these analytes are not biologically relevant.

Summary of Compositional Analyses

Pioneer highlights that the genetic modification does not meaningfully affect nutrient composition and nutritional value of forage and grain derived from DP202216 corn. Pioneer concludes that DP202216 corn is comparable to corn varieties that are currently used in animal food in the United States.

Conclusion

CVM evaluated Pioneer's submissions to determine whether DP202216 corn raises any safety or regulatory issues with respect to its use in animal food. Based on the information provided by Pioneer and other information available to the agency, CVM did not identify any safety or regulatory issues under the FD&C Act that would require further evaluation at this time.

Pioneer concludes that DP202216 corn and the animal foods derived from it are as safe as and are not materially different in composition or any other relevant parameter from other corn varieties now grown, marketed, and consumed. At this time, based on Pioneer's data and information, CVM considers Pioneer's consultation on DP202216 corn for use in animal food to be complete.

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