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CENTER FOR VETERINARY MEDICINE

Biotechnology Notification File No. 000204
CVM Note to the File

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From: Jing Ning, Ph.D.

To: Administrative Record, BNF No. 000204

Subject: Event MON 96012 Cotton

Keywords: Cotton, *Gossypium hirsutum* (L.), 5-enolpyruvylshikimate-3-phosphate synthase, *cp4 epsps* gene, *Agrobacterium sp*. strain CP4, Phosphinothricin N-acetyltransferase, *pat* gene, Dicamba mono-oxygenase, *dmo* gene, *Strenotrophomonas maltophilia*, Protoporphyrinogen IX oxidase, *H_N90 PPO* gene, *Enterobacter cloacae*, Triketone dioxygenase, *TDO* gene, *Oryza sativa*, Tolerance to glyphosate, glufosinate, dicamba, mesotrione, and PPO-inhibiting herbicides, herbicide tolerance, OECD identifier MON-96Ø12-6, Bayer CropScience, LP.

Purpose

This document summarizes the Food and Drug Administration (FDA) Center for Veterinary Medicine's (CVM, we) evaluation of biotechnology notification file (BNF) number 000204. Bayer CropScience LP (Bayer) submitted a safety and nutritional assessment for a genetically engineered (GE) cotton, transformation event MON 96012 (hereafter referred to as MON 96012 cotton), and additional information afterwards. CVM evaluated the information in Bayer's submissions to ensure that regulatory and safety issues regarding animal food derived from MON 96012 cotton have been resolved prior to commercial distribution. FDA's Human Foods Program summarizes its evaluation of uses of MON 96012 cotton in human food in a separate document.

In CVM's evaluation, we considered all of the information provided by Bayer 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 intended effects of the modifications in MON 96012 cotton are to confer tolerance to several herbicides. Bayer states that the parental cotton variety was transformed with the *dicamba mono-oxygenase* (*dmo*) gene from *Stenotrophomonas maltophilia*, which encodes the DMO protein that confers tolerance to dicamba herbicide. To confer tolerance to glyphosate, Bayer introduced the *aroA* gene from *Agrobacterium* sp. strain CP4 (*cp4 epsps* gene) that encodes CP4 5-enolpyruvylshikimate-3-phosphate synthase

(CP4 EPSPS). Additionally, MON 96012 cotton expresses a copy of the *phosphinothricin N-acetyltransferase* (*pat*) gene from *Streptomyces viridochromogenes*, which encodes the PAT protein that confers tolerance to glufosinate herbicide. Bayer also introduced the *triketone dioxygenase* (*TDO*) gene, derived from the *Oryza sativa*, that expresses the TDO protein to confer tolerance to mesotrione herbicide. Bayer introduced the *protoporphyrinogen IX oxidase* (*H_N90 PPO*) gene from *Enterobacter cloacae*, which encodes the PPO protein that confers tolerance to PPO-inhibiting herbicides.

Regulatory Considerations

The purposes of this evaluation are (1) to assess whether Bayer 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).

Genetic Modification and Characterization

Introduced DNA and Transformation Method

Bayer transformed meristem explants obtained from a non-genetically engineered cotton variety DP393 with vector PV-GHHT529207 using disarmed *Agrobacterium tumefaciens* mediated transformation. Bayer states that the transfer-DNA (T-DNA) region within the plasmid contained seven expression cassettes between the left (LB) and right (RB) border sequences. These include:

- *aadA* selectable marker cassette.
- *cre* recombinase cassette.
- *pat* gene from *S. viridochromogenes* that was codon optimized for expression in cotton, which is preceded by promoter and 5' (untranslated region) UTR sequence of the Rubisco from *Arabidopsis thaliana* and followed by 3´UTR sequence of the *Hsp20* gene from *Medicago truncatula*.
- *TDO* gene derived from the *Oryza sativa* that was codon optimized for expression in cotton, which is preceded by promoter and 5' UTR sequence of a *chlorophyll a/b binding* gene and followed by a 3´UTR developed based on sequences from *M. truncatula*.
- *dmo* gene from *S. maltophilia* that was codon optimized for expression in cotton, which is preceded by the promoter region of the polyubiquitin gene (ubq10) from *Arabidopsis thaliana* and chloroplast targeting sequence of the *albino or pale green 6* gene from *A. thaliana*. Following the *dmo* gene is the 3´ UTR sequence of the *sali3-2* gene from *M. truncatula*.
- *cp4 epsps* gene from *Agrobacterium,* which is preceded by promoter, 5´ UTR and intron sequences of the EF-1 alpha gene from *A. thaliana* and a chloroplast target sequence from the *Waxy* gene of *Triticum aestivum L.* Following the *cp4 epsps* gene is the 3´ UTR sequence of the *rbcS* gene from *Pisum sativum*.
- *H_N90 PPO* gene from *E. cloacae*, which is preceded by the enhancer, promoter, 5´UTR from *A. thaliana* and chloroplast targeting sequence of the *albino or pale green 6* gene from *A. thaliana*. Following the *H_N90 PPO* gene is the 3´UTR sequence from cotton.

The first two cassettes are flanked by *loxP* excision targeting sequences. Following transformation, meristems were grown on selection media[1], and developed plants were selected as the R0 generation. After a successful transformant was selected, the *aadA* selectable marker cassette and the *cre* cassette were excised through Cre/lox-mediated auto-excision from the R0 generation. These plants were self-pollinated and the R1 population was screened for the presence of T-DNA, absence of vector backbone sequences and phenotypic assessments.

Bayer characterized the number of T-DNA inserts, the number of insert junctions, the absence of vector backbone sequences, and organization and intactness of each insert in MON 96012 cotton using whole genome sequencing (WGS) and overlapping PCR. The conventional control, DP393, was used as the comparator in these analyses. Bayer reports a minimum average read depth of 98-fold. Bayer reports that a single copy of the T-DNA sequence was inserted into the cotton genome and that MON 96012 cotton does not contain sequences from the vector backbone. Directed DNA sequencing demonstrated that the genetic elements within the inserted T-DNA were intact. A sequence comparison between the PCR product generated from the control and the sequence generated from the 5´ and 3´ flanking sequence of MON 96012 cotton indicated that the T-DNA insert replaced 108 bp of cotton genomic DNA. Bayer states that these types of changes are probably the result of double stranded break repair during the *Agrobacterium*-mediated transformation process.

The stability of the inserted T-DNA sequences in MON 96012 cotton across multiple breeding generations and the comparator DP393 were evaluated by WGS followed by bioinformatics analyses. Bayer reports that the single locus of integration that was characterized in the R3 generation of MON 96012 cotton is present in all five breeding generations of the MON 96012 cotton. Within three back-crossed generations, Bayer collected genotypic segregation data to assess inheritance pattern, and these data were analyzed using Chi square analysis. Bayer concludes that the inserted DNA segregated as a single locus in accordance with the principles of Mendelian inheritance.

Bayer performed bioinformatics analyses using the nucleotide sequences obtained for the T-DNA insert and junction sequences to determine whether insertion of the introduced DNA created any potential open reading frames (ORF) that could encode for putative polypeptides. This analysis included nucleotide sequences for each of the six reading frames. Bayer evaluated the putative polypeptides against the TOX_2024 database[2] to determine the similarity of these putative polypeptides to known toxins. Bayer reports that none of the putative polypeptides had significant identity with proteins in its toxin database, TOX_2024. Based on the results of bioinformatics analyses, Bayer concludes that the T-DNA insertion does not lead to the production of putative polypeptides that would raise animal food safety concerns.

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[1] The selection media contained spectinomycin and streptomycin for selection of putative transformants, carbenicillin disodium salt and cefotaxime sodium salt to eliminate Agrobacterium.

[2] According to Bayer, TOX_2024 is a subset of sequences derived from the Swiss-Prot database that was selected using a keyword search and filtered to exclude non-toxin proteins.

Protein Safety

Bayer highlights that the PAT, CP4 EPSPS, DMO and TDO proteins expressed in MON 96012 cotton are identical to the proteins present in BNFs that have completed FDA's Biotechnology Consultations on Food from GE Plant Varieties and includes by reference information on safety of these proteins in these crops.[3] Bayer notes that the conclusions of the multi-step approach to the safety assessments described in previous consultations, including oral acute toxicology, digestibility, heat susceptibility, and sequence similarities to known toxins through bioinformatics analysis, are applicable to the PAT, CP4 EPSPS, DMO and TDO proteins expressed in MON 96012 cotton.

MON 96012 cotton was genetically engineered to express HemG-type PPO protein derived from *E. cloacae*. HemG-type PPO is a smaller PPO enzyme that catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX, thereby conferring tolerance to PPO-inhibiting herbicides in MON 96012 cotton by maintaining the oxidation of protoporphyrinogen IX to protoporphyrin IX.

To conduct safety assessment of PPO protein, Bayer produced the PPO protein in *E. coli*. Bayer confirmed the identity and biochemical equivalence of the PPO protein expressed in *E. coli* and MON 96012 cotton, by comparing results from amino acid sequence, molecular weight and immunoreactivity, glycosylation, and functional activity.

Bayer used a weight of evidence approach to assess the safety of the PPO protein. Bayer highlights that the donor organism, *E. cloacae*, is a common soil, water, plants, and the gastrointestinal tracts bacterium that is ubiquitously present in the environment. Bayer demonstrated that the PPO protein was rapidly digested with pepsin and pancreatin and rapidly lost activity at 55 °C. Bayer carried out bioinformatics analysis and conclude that PPO protein in MON 96012 cotton does not share similarity with any sequences in the TOX_2024 database. Bayer states that there was no evidence of acute toxicity in mice that were dosed orally at 5000 milligrams of PPO protein/kilogram of body weight. Taken together, Bayer concludes that dietary exposure to PPO protein in MON 96012 cotton poses no meaningful risk to animal health.

Protein Expression Level

Bayer quantified CP4 EPSPS, PAT, DMO, TDO, and PPO protein levels in various tissues of MON 96012 cotton. The study included five field trial sites with four replicate plots at each site. Cottonseed, pollen, boll, square, leaf, and root samples were collected from each replicated plot at all field sites treated with glyphosate, glufosinate, dicamba, mesotrione, and flumioxazin herbicides. Samples were prepared and analyzed using

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[3] Bayer notes that PAT protein produced in MON 96012 cotton is identical to the PAT protein expressed in MON 88701 cotton (BNF No. 000135), MON 87419 corn (BNF No. 000148), MON 87429 corn (BNF No. 000173) and MON 94313 soybean (BNF No. 000193). Bayer notes that CP4 EPSPS protein produced in MON 96012 cotton is identical to the CP4 EPSPS protein expressed in MON 88302 canola (BNF No. 000127), MON 87429 corn (BNF No. 000173) and other completed BNFs. Bayer notes that DMO and TDO proteins produced in MON 96012 cotton are identical to the DMO and TDO proteins in MON 94313 soybean (BNF No. 000193).

Enzyme-linked immunosorbent assay.

Bayer report that means CP4 EPSPS level in MON 96012 cotton across all sites was highest in over-season leaf (OSL)1 at 230 micrograms/gram of tissue dry weight (DW) and lowest in pollen at 3.0 micrograms/gram DW. Mean PAT protein level in MON 96012 cotton across all sites was highest in OSL1 at 34 micrograms/gram DW and lowest in pollen at < limit of quantitation (LOQ). Mean DMO protein level in MON 96012 cotton across all sites was highest in OSL4 at 260 micrograms/gram DW and lowest in boll at 13 micrograms/gram DW. Mean TDO protein level in MON 96012 cotton across all sites was highest in OSL1 at 60 micrograms/gram DW and lowest in pollen at < LOQ. Mean PPO protein level in MON 96012 cotton across all sites was highest in square at 27 micrograms/gram DW and lowest in over season root 3 at 0.37 micrograms/gram DW.

Animal Food Use

The developer states that MON 96012 cotton is expected to be grown for the same uses as currently commercialized cotton, and no new uses in animal food are anticipated. Bayer references the Organisation for Economic Co-operation and Development (OECD) consensus document on biology of cotton and the use of cotton as a crop plant.[4]. Following cleaning and ginning, whole cottonseed (after removal of the cotton fibers) is processed into four major human and animal food products: oil, linters, meal, and hulls. The first two ingredients are almost exclusively used in human food. Whole cottonseed, acid delinted cottonseed, cottonseed meal, hulls, and cotton gin trash are used as ingredients in food for ruminant animals.[5] The amount of cottonseed meal that can be used in monogastric animal diets is normally limited by the presence of gossypol. Cottonseed meal which contains not more than 0.04% (400 parts per million (ppm)) free gossypol, can be used as a source of protein in food for animals. For example, cottonseed meal from glandless varieties of cotton have been used in animal food for monogastric species, such as swine and poultry, and aquaculture.

Composition

Scope of Analysis

Bayer analyzed the nutrient composition of MON 96012 cotton and DP393 (conventional control) cotton that were grown and harvested under similar conditions. Compositional analyses of cottonseed samples were reported for components listed in the OECD cotton composition consensus document.

Study Design

Bayer conducted field trials in 2023 at five locations in the United States. A randomized complete block design with four replicate plots was used at each field site. Bayer harvested cottonseed samples from each replicate within each site for composition analysis. Cottonseed samples were harvested at physiological maturity and shipped at

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[4] Organisation for Economic Co-operation and Development. 2009. Consensus document on the compositional considerations for new varieties of cotton (*Gossypium hirsutum* and *Gossypium barbadense*): Key food and feed nutrients and anti-nutrients. ENV/JM/MONO (2004)16. OECD, Paris

[5] NCPA. 2002. Cottonseed and its products. National Cottonseed Products Association, Cordova, Tennessee. http://www.cottonseed.com/publications/cottonseedanditsproducts.asp.

ambient temperature from the field sites to Bayer (where they were ginned). The samples were subsequently acid-delinted and a subsample for compositional analysis was obtained from each tissue sample collected. These subsamples were ground and stored in a freezer at -20°C. The samples were shipped on dry ice to analytical lab for nutrient compositional analysis.

Components that were analyzed in cottonseed samples included proximates (crude protein, crude fat, ash and carbohydrates by calculation[6]), fiber (acid detergent fiber (ADF), total dietary fiber (TDF), and neutral detergent fiber (NDF)), 18 amino acids, seven fatty acids, two minerals, vitamin E, dihydrosterculic acid, free gossypol, malvalic acid, sterculic acid and total gossypol. Results were all expressed on a dry matter basis prior to statistical analyses. Moisture values were not statistically analyzed. Bayer statistically compared each component for MON 96012 cotton with the control across-locations using a linear mixed model with site and replicate as random factors. T-test analyses were used to test at the level of $p \leq 0.05$ for differences between MON 96012 cotton and control. Differences between MON 96012 cotton and control were evaluated in context of variation within the control germplasm grown across multiple sites and of natural variability defined by values for cotton varieties in the Agriculture and Food Systems Institute Crop Composition Database (AFSI-CCDB)[7] and in the scientific literature.

Results of Analyses

Bayer reports statistically significant differences between MON 96012 cotton and the control in the levels of 11 components (arginine, total fat, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linolenic acid, phosphorus and dihydrosterculic acid). However, Bayer notes that the mean values for all of these components were within the range of values observed in the AFSI-CCDB and/or scientific literature. Bayer concludes that the observed statistically significant differences between MON 96012 cotton and the control are not biologically meaningful from an animal food safety perspective.

Summary of Compositional Analyses

Bayer states that expression of the five proteins that impart tolerance to different herbicides does not meaningfully alter the nutrient composition of MON 96012 cotton. Bayer concludes that these results support the conclusion that cottonseed obtained from MON 96012 cotton is compositionally equivalent to the control in the levels of key nutrients and anti-nutrients.

Conclusion

CVM evaluated Bayer's submissions to determine whether MON 96012 cotton raises any safety or regulatory issues with respect to its use in animal food. Based on the information provided by Bayer and other information available to the agency, CVM did

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[6] Percent carbohydrates = 100% - (% protein + % fat + % moisture + % ash).

[7] Agriculture and Food Systems Institute (AFSI) Crop Composition Database: https://www.cropcomposition.org.

not identify any safety or regulatory issues under the FD&C Act that would require further evaluation at this time.

Bayer concludes that MON 96012 cotton 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 cotton varieties now grown, marketed, and consumed. At this time, based on Bayer's data and information, CVM considers Bayer's consultation on MON 96012 cotton for use in animal food to be complete.

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