Biotechnology Consultation - Note to File
Biotechnology Notification File No. 000157

Date

October 10, 2017

Subject

Male sterile and herbicide tolerant MS11 canola

Keywords

Male sterile, MS11, canola, *Brassica napus,* BNF57, MS8, Barnase, *Bacillus amyloliquefaciens,* Barstar, *bar,* Phosphinothricin acetyltransferase (PAT), *Streptomyces hygroscopicus,* herbicide tolerance, glufosinate-ammonium, OECD unique identifier BCS-BNØ12-7, Bayer CropScience LP

Purpose

This document summarizes the Food and Drug Administration's (FDA's, our) evaluation of biotechnology notification file (BNF) No. 000157. Bayer CropScience LP (Bayer) submitted a safety and nutritional assessment of genetically engineered herbicide tolerant and male sterile canola, transformation event MS11 (MS11 canola), which we received on August 26, 2016. Bayer provided additional information on February 21, 2017 and February 22, 2017. We evaluated the information in Bayer's submissions to ensure that regulatory and safety issues regarding human or animal food derived from MS11 canola have been resolved prior to commercial distribution.

In our evaluation, we considered all 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, but do not intend to restate the information provided in the final consultation in its entirety.

Intended Effects

The intended effect of the modifications in MS11 canola is male sterility, which facilitates cross-pollination. Bayer intends to use MS11 canola for cross-pollination with the fertility restorer line RF3[1] canola to produce F1 hybrid seed.

A second intended effect of the modifications in MS11 canola is tolerance to the herbicide glufosinate-ammonium. Bayer used herbicide tolerance to select for transformed plants. Bayer also intends to use the herbicide tolerance trait to select for and maintain the MS11 canola line.

[1] Fertility restorer RF3 canola and a male sterile line, MS8 canola, were the subjects of BNF No. 000057.

To confer male sterility, Bayer introduced the coding sequence of the *barnase* gene, which encodes a ribonuclease, Barnase. Barnase expression is restricted to the tapetum, a layer of cells surrounding the pollen sac, where Barnase interferes with pollen development and results in a lack of viable pollen. Bayer also introduced the coding sequence of the *barstar* gene, encoding the Barstar protein, which inhibits Barnase. MS11 canola expresses Barstar, which was intended to enhance transformation efficiency. To confer herbicide tolerance, Bayer introduced the *bar* gene, which encodes a phosphinothricin acetyltransferase (PAT/*bar*).

Regulatory Considerations

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

The United States 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 and metabolic by-products in MS11 canola are considered pesticide residues.

Genetic Modification and Characterization

Transformation Plasmid and Method

Bayer transformed hypocotyl segments from seedlings of *Brassica napus* variety N90-740 with transformation plasmid pTCO113 using *Agrobacterium tumefaciens*.

The transfer DNA (T-DNA) region in plasmid pTCO113 contains three expression cassettes:

1. A *barnase* expression cassette consisting of the anther-specific promoter of the *TA29* gene from *Nicotiana tabacum*, the *barnase* coding sequence[2] and its 3′ untranslated region from *Bacillus amyloliquefaciens*, and the 3′ untranslated region of the nopaline synthase (*nos*) gene from *A. tumefaciens*.

2. A *barstar* expression cassette consisting of the promoter from the *nos* gene from *A. tumefaciens*, the *barstar* coding sequence from *B. amyloliquefaciens*, and the 3′ untranslated region of TL-DNA gene 7 from the *A. tumefaciens* octopine Ti plasmid.

3. A *bar* expression cassette consisting of the promoter of the ribulose-1,5-biphosphate carboxylase small subunit gene from *Arabidopsis thaliana*, the *bar*

[2] Bayer notes that minor amino acid substitutions were engineered into the *barnase* coding sequence.

gene from *Streptomyces hygroscopicus*[3] encoding a PAT protein[4], and the 3′ untranslated region of TL-DNA gene 7 from the *A. tumefaciens* octopine Ti plasmid.

Following transformation, Bayer cultured callus from wounded hypocotyl tissue and generated plantlets. Bayer used glufosinate-ammonium to select for plants expressing the *bar* gene. Bayer then cross-pollinated glufosinate-ammonium-tolerant plants with untransformed N90-740 canola for two generations and assessed insertion events for the presence of the insertion sequence, copy number, insertion integrity, and absence of vector backbone.

Characteristics, Inheritance, and Stability of the Introduced DNA

Bayer characterized the inserted DNA in MS11 canola by Southern blot analysis and by amplifying the inserted DNA and flanking genomic DNA using polymerase chain reaction (PCR) followed by direct sequencing. Southern blot analysis demonstrated the presence of one complete DNA insert containing the *barnase*, *barstar,* and *bar* genes. Sequencing was used to compare the inserted DNA to the sequence of the pTCO113 plasmid and to analyze the flanking DNA. Bayer identified a 40-base pair deletion of genomic DNA at the insertion site with no rearrangements in the regions flanking the insertion. Based on bioinformatics analysis, Bayer does not expect the insertion to interrupt or alter the expression of known canola genes.

Southern blot analysis using five probes and PCR analysis targeting four distinct elements of the vector demonstrated the absence of vector backbone[5] sequences in MS11 canola.

Bayer demonstrated the stability of the inserted DNA by showing consistent Southern blot hybridization results over five generations. Bayer used PCR to determine the segregation ratios of the inserted DNA and Chi-square analysis to show that the insertion in MS11 canola is inherited according to Mendelian principles, in a way that is consistent with a single, chromosomal insertion.

Using bioinformatics analyses, Bayer assessed putative translation products of open reading frames (ORFs) generated by the inserted DNA and junctions for homology to known toxic or allergenic proteins. Bayer reports that none of the putative ORFs 30 amino acids or longer would encode peptides with similarity to known allergens in the public allergen database AllergenOnline (FARRP) or known toxins in the NCBI non-redundant protein database.

[3] Bayer notes that minor amino acid substitutions were engineered into the *bar* coding sequence.
[4] Referred to as PAT/*bar*; a similar protein is expressed from the *Streptomyces viridochromogenes pat* gene.
[5] The vector backbone is the region of pTCO113 outside of the T-DNA.

Protein Characterization

Identity and Function of Introduced Proteins

The Barnase protein from *B. amyloliquefaciens* is a ribonuclease (RNase), a type of enzyme that catalyzes the cleavage of ribonucleic acid (RNA). RNases play a central role in cellular RNA metabolism in both prokaryotes and eukaryotes. Barnase is a protein of 111 amino acids with a predicted molecular weight of 12.5 kilodaltons (kDa).

The Barstar protein from *B. amyloliquefaciens* binds to the Barnase protein with high specificity and inhibits its activity. The only known function of Barstar is to inhibit Barnase. Barstar is a protein of 90 amino acids with a predicted molecular weight of 10.3 kDa.

The PAT/*bar* protein from *S. hygroscopicus* is an acetyltransferase composed of 183 amino acids with a predicted molecular weight of 20.7 kDa. PAT/*bar* confers tolerance to the herbicide glufosinate-ammonium by acetylating the active ingredient, L-phosphinothricin and thereby inactivating it.

Protein Expression Level

Bayer used sandwich enzyme-linked immunosorbent assays (ELISA) to quantify the expression of Barnase, Barstar, and PAT/*bar* in MS11 canola grown in 2014 under typical commercial production conditions at two sites in Canada and at one site in the United States. At each site, one plot was treated with glufosinate-ammonium and one plot was left untreated. Barnase and Barstar were below the lower limit of quantification (LLOQ) in grain.[6] The mean PAT/*bar* concentration was 0.49 ± 0.18 µg/g dry weight in grain from treated plants and 0.34 ± 0.18 µg/g dry weight in grain from untreated plants.

Potential for Toxicity and Allergenicity of the Introduced Proteins

Bayer notes that the safety of Barnase, Barstar, and PAT/*bar* were discussed in BNF 000032 and BNF 000057, and that PAT proteins have been evaluated in a total of 26 completed consultations. Since the previous evaluations, Bayer conducted additional studies on the safety of the Barnase, Barstar, and PAT/*bar*, which Bayer summarizes. The additional data Bayer provides are consistent with the previous safety conclusions.

Bayer repeated bioinformatics analysis of the three proteins to assess whether they are similar to known toxins or allergens. Bayer found no structurally relevant similarity with entries in the public allergen database AllergenOnline (FARRP), known toxins in the NCBI non-redundant protein database, and Bayer's in-house protein toxin database.

Bayer conducted acute oral toxicity studies in mice administered microbially produced Barnase, Barstar, and PAT/*bar*. Each study found no adverse effects up to and including the highest tested level.

[6] Presence of Barnase in grain would not be expected because its expression is driven by an anther-specific promoter.

Bayer assessed the heat stability of each protein. Consistent with previous studies, PAT/*bar* remained detectable but lost enzymatic activity after incubation at 90°C for 60 minutes. Barnase underwent degradation at temperatures 55°C and higher after 30 minutes and lost activity at temperatures above 55°C. Barstar underwent partial degradation and lost activity at 75°C and 95°C.

Bayer assessed the stability of each protein in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). PAT/*bar* degraded rapidly in both SGF and SIF. Barnase degraded quickly in SGF, but degraded less quickly in SIF. Barstar degraded rapidly in SGF, and 90% of Barstar was degraded within 10 minutes in SIF.

Bayer concludes that collectively the updated evaluations of PAT/*bar*, Barnase, and Barstar are consistent with previous safety conclusions and reconfirm the safety of these proteins.

Human and Animal Food Use

Canola (*B. napus* or *B. rapa*) refers to rapeseed varieties low in erucic acid and glucosinolates. Canola is used primarily to produce oil for human food.[7] Canola oil is low in saturated fatty acids and is commonly used as cooking oil for frying, baking, and other food applications. Canola meal is a byproduct of oil crushing. The majority of canola meal is used in animal food, primarily for cattle and pigs, and, to a lesser extent, poultry, aquaculture, lamb, and other livestock. Industrial uses of canola are limited.

Composition

Scope of Analysis

Bayer analyzed the composition of MS11 canola grain, including nutrients, antinutrients, and toxicants. Bayer compared measurements from MS11 canola to the non-transformed recipient variety N90-140 (control) and to six non-genetically engineered commercial reference varieties grown concurrently (three varieties were grown at each site).

Study Design - Compositional Analyses

Bayer produced samples for compositional analysis in 2015 at nine sites in canola-growing regions of the United States and Canada selected to represent the range of environments where canola is grown. MS11 canola was grown both with glufosinate-ammonium treatment (treated MS11 canola) and without (untreated MS11 canola). Four replicates of each entry were grown at each site with randomized complete block design.

For statistical analysis, Bayer omitted components if over one third of the values were below the limit of quantification (LOQ). If fewer than one third of values were below the LOQ, Bayer replaced values below the LOQ with a value equal to half of the LOQ.

[7] To date, FDA has evaluated and responded to two GRAS (generally recognized as safe) notices on the use in human food of canola protein isolates (GRAS Notices GRN 000327 and GRN 000386). FDA had no questions about the notifiers' conclusions that the intended uses of canola protein isolates in human food are GRAS.

Concentrations of each component were expressed on a dry matter basis prior to statistical analysis.

Composition data for each component from treated MS11 canola, untreated MS11 canola, and control were analyzed using combined and individual site, mixed model analysis of variance approaches. Bayer compared levels of each component in both treated and untreated MS11 canola to the control using t-tests at a level of $p < 0.05$. Bayer calculated tolerance intervals for each component from the data obtained from reference varieties.[8] The ranges and tolerance intervals for the reference varieties were used by Bayer to assess whether differences in nutrient composition were biologically relevant.

Results of analyses

Bayer measured proximates (moisture, crude protein, crude fat, ash, carbohydrate by calculation, acid detergent fiber, and neutral detergent fiber), vitamins (vitamin E and Vitamin K1), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), 18 amino acids, 34 fatty acids, 14 glucosinolates, phytic acid, tannins (soluble and insoluble), and sinapine. Bayer lists 35 components it excluded from statistical analysis because over one third of the values were below the LOQ.

Based on the analyses of the combined-site data, Bayer found statistically significant differences between the control and untreated MS11 canola in the levels of gluconapin (a glucosinolate) and insoluble tannins. Bayer observed statistically significant differences between the control and treated MS11 canola in the levels of 30 components. However, in all instances, the levels of components in treated and untreated MS11 canola fell within the range of the reference varieties and within the tolerance intervals.[9] Bayer explains that compositional differences between the control and herbicide-treated MS11 canola are expected because MS11 canola plants that survive herbicide treatment are male-sterile and produce seed through cross-pollination.

Summary of Compositional Analyses

Bayer states that statistically significant differences observed between MS11 canola and the control are not biologically relevant because the means for all measured components in MS11 canola fell within the range of values for the reference varieties. Bayer concludes that grain from MS11 canola is compositionally equivalent to grain from the reference varieties.

Conclusion

FDA evaluated Bayer's submission to determine whether MS11 canola raises any safety or regulatory issues with respect to its uses in human or animal food. Based on the information provided by Bayer and other information available to the agency, FDA did not identify any safety or regulatory issues under the Federal Food, Drug and Cosmetic Act that would require further evaluation at this time.

[8] The tolerance interval calculated by Bayer represents, with 95% confidence, 99% of the population of values from the reference varieties.

[9] Results from individual site data supported the conclusions from the combined-site data.

Bayer has concluded that its male-sterile and herbicide tolerant canola variety, MS11 canola, and the human and animal foods derived from it, are as safe as and are not materially different in composition or any other relevant parameter from other canola varieties now grown, marketed, and consumed. At this time, based on Bayer's data and information, the agency considers Bayer's consultation on MS11 canola to be complete.

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