U.S. FOOD & DRUG ADMINISTRATION

Human Foods Program

Biotechnology Notification File No. 000198
HFP Note to the File

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To: Administrative Record, BNF No. 000198

Subject: Sugar beet with transformation event KWS20-1 (KWS20-1 sugar beet)

Keywords: Sugar beet, *Beta vulgaris*, herbicide tolerance, dicamba, demethylase (*dmo*) gene, dicamba mono-oxygenase, DMO, *Stenotrophomonas maltophilia*, glufosinate, *pat* gene, phosphinothricin-N-acetyltransferase, PAT, *Streptomyces viridochromogenes*, glyphosate, *aroA* gene (*cp4 epsps* gene), CP4 5-enolpyruvylshikimate-3-phosphate synthase, CP4 EPSPS, *Agrobacterium* sp. strain CP4, Bayer CropScience LP, KWS SAAT SE & Co. KGaA, KWS20-1, OECD Unique Identifier KB-KWS2Ø1-6

Summary

Bayer CropScience LP (Bayer) and KWS SAAT SE & Co. KGaA (KWS) have completed a consultation with the Food and Drug Administration (FDA) on food derived from KWS20-1 sugar beet with multiple herbicide tolerance traits.[1] KWS20-1 expresses dicamba mono-oxygenase (DMO) for tolerance to dicamba herbicide; phosphinothricin-N-acetyltransferase (PAT) for tolerance to glufosinate-ammonium herbicide; and CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) for tolerance to glyphosate herbicide. This document summarizes Bayer and KWS's conclusions and supporting data and information that FDA's Human Foods Program (HFP, we) evaluated pertaining to human food uses. FDA's Center for Veterinary Medicine summarizes its evaluation pertaining to animal food uses in a separate document.

Based on the safety and nutritional assessment Bayer and KWS have conducted, it is our understanding that Bayer and KWS conclude:

- they have not introduced into human food a new protein or other substance that would require premarket approval as a food additive;
- human food from KWS20-1 sugar beet is comparable to and as safe as human food from other conventional sugar beet varieties.

HFP evaluated data and information supporting these conclusions and considered whether KWS20-1 sugar beet raises other regulatory issues involving human food within FDA's authority under the Federal

[1] The United States Environmental Protection Agency (EPA) registers pesticides (including herbicides) under the Federal Insecticide, Fungicide, and Rodenticide Act. Under the Federal Food, Drug, and Cosmetic Act, EPA establishes tolerances (maximum legally permissible levels) of residues of pesticides in food.

Food, Drug, and Cosmetic Act. We have no further questions at this time about the safety, nutrition, and regulatory compliance of human food from KWS20-1 sugar beet.

Subject of the Consultation

Crop	Sugar beet (*Beta vulgaris*)
Designation	KWS20-1
Intended trait	Tolerance to dicamba herbicide
Intended trait	Tolerance to glufosinate-ammonium herbicide
Intended trait	Tolerance to glyphosate herbicide
Developer	Bayer CropScience LP and KWS SAAT SE & Co. KGaA
Submission received	June 6, 2023
Amendment(s) received	June 7, 2024; November 26, 2024
Intended use	General use in human foods
Transformation plasmid	PV-BVHT527462
Expression cassette 1	The *dmo* expression cassette contains a codon-optimized demethylase (*dmo*) gene from *Stenotrophomonas maltophilia* encoding dicamba mono-oxygenase (DMO) protein.
Expression cassette 2	The *pat* cassette contains a codon-optimized phosphinothricin-N-acetyltransferase (*pat*) gene from *Streptomyces viridochromogenes* encoding phosphinothricin-N-acetyltransferase (PAT) protein.
Expression cassette 3	The *cp4-EPSPS* cassette contains a codon-optimized *aroA* gene (*cp4 epsps* gene) from *Agrobacterium* sp. strain CP4 encoding 5-enolpyruvylshikimate-3- phosphate synthase (CP4 EPSPS) protein.
Method for conferring genetic change	*Agrobacterium tumefaciens*-mediated transformation

Molecular Characterization

Confirmation of intended genetic change

Bayer and KWS developed KWS20-1 sugar beet lines by introducing the T-DNA harboring *dmo*, *aroA,* and *pat* genes and regulatory elements into calli of a conventional sugar beet variety that has a history of safe use through *Agrobacterium* mediated transformation. Transformed callus tissues showing resistance to phosphinothricin in the selection media were used to regenerate shoots. Regenerated shoots were transferred to propagation plugs for root development. Rooted plants that showed normal phenotype were selected for molecular characterization. Bayer and KWS used a combination of Southern blotting,

Polymerase Chain Reaction (PCR), Kompetitive Allele Specific PCR (KASP)[2] and sequencing to confirm the integrity and copy number of the DNA insertion. Bayer and KWS identified two insertion junctions that revealed the presence of a single T-DNA insertion in the genome. Bayer and KWS amplified the insertion and flanking genomic sequences using PCR and conducted sequencing analysis. From the sequencing results, Bayer and KWS concluded that the KWS20-1 sugar beet genome contains a single, intact copy of the T-DNA harboring all three genes and regulatory elements. Comparison of the sequence to that from the conventional sugar beet variety revealed a seven base pair deletion of sugar beet genomic DNA at the T-DNA insertion site.

Absence of vector backbone DNA

Bayer and KWS conducted Southern blot for the detection of vector backbone sequences in the genome of KWS20-1. They did not detect any backbone sequences.

Inheritance and stability

Bayer and KWS performed Southern blot analysis on three generations of KWS20-1 sugar beet to confirm the inheritance and stability of the insert. Bayer and KWS found consistent hybridization patterns in each generation, confirming the stable inheritance of the T-DNA insertion. Bayer and KWS performed segregation analysis by using Chi square analysis across generations of KWS20-1 sugar beet to determine the inheritance pattern of the inserted T-DNA. The Chi square value indicated no statistically significant difference between the observed and expected segregation ratios of KWS20-1 sugar beet. Bayer and KWS concluded that the KWS20-1 sugar beet T-DNA resides at a single locus and is inherited according to Mendelian principles of inheritance.

Open reading frame analysis

Bayer and KWS carried out bioinformatics analysis to assess whether new open reading frames (ORFs) were generated as a result of the T-DNA insertion in KWS20-1 sugar beet and, if so, whether the putative translation of new ORFs raised allergenicity or toxicity concerns relevant to human food safety. Bayer and KWS assessed new ORFs formed across the junctions between inserted T-DNA and sugar beet genomic DNA by surveying all six reading frames and translating the corresponding sequences from stop codon to stop codon. Bayer and KWS reported that none of the putative, corresponding polypeptides (eight amino acids or greater in length) of the hypothetical new ORFs from KWS20-1 sugar beet showed significant sequence similarity (*E*-score $<1 \times 10^{-5}$ was deemed significant) with the sequences of known allergens (AD_2022 database)[3], toxins (TOX_2022 database)[4] or other biologically active proteins (PRT_2022 database)[5] that could affect human or animal health. Bayer and KWS also used sequence alignment tool to examine each putative polypeptide for structural similarities with known allergens and toxins by searching databases.[6] Bayer and KWS reported that no structural similarities between the putative polypeptides and known allergens, toxins, or biologically active proteins were found.

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[2] Semagn K, et al., (2013): Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Molecular Breeding 33: 1-14.

[3] The allergen, gliadin and glutenin proteins sequence database (AD_2022) was derived from the "COMprehensive Protein Allergen REsource" (COMPARE) database from the Health and Environmental Sciences Institute (February 2, 2022).

[4] The UniProt toxin protein database (TOX_2022) is a subset of sequences derived from the Swiss-Prot database (found at https://www.uniprot.org/) that were selected using a keyword search and filtered to remove likely non-toxin proteins (January 12, 2022).

[5] The PRT_2022 database is a collection of the full GenBank proteins downloaded from NCBI on January 10, 2022.

[6] The structural similarities were determined by detailed visual inspection of the alignment, calculated percent identity, alignment length, and *E*-score ($<1 \times 10^{-5}$ was deemed significant).

Introduced Protein: dicamba mono-oxygenase (DMO)

Intended trait	Tolerance to dicamba herbicide
Source organism	*Stenotrophomonas maltophilia*
Protein description	DMO expressed in KWS20-1 sugar beet was identical to DMO from *Stenotrophomonas maltophilia,* except for an additional leucine at position 2 and the addition of a chloroplast-targeting peptide at the N terminus.
Intended function	DMO catalyzes the demethylation of dicamba to the non-herbicidal compounds 3,6-dichlorosalicylic acid and formaldehyde.

Bayer and KWS used an enzyme linked immunosorbent assay (ELISA) to measure the level of DMO protein in KWS20-1 sugar beet root. The mean level of DMO in root was detected at a range from 12-28 μg/g dry weight. Bayer and KWS conclude that the concentration of DMO in sugar beet root tissues is very low and cite a publication[7] documenting that refined sugar from sugar beets does not contain detectable levels of protein.

FDA previously evaluated DMO in consultations BNF 000125, BNF 000135, BNF 000148, BNF 000173, and BNF 000177. Bayer and KWS include information from these previous consultations by reference. Bayer and KWS also refer to published safety assessments of DMO, which cite studies on acute oral toxicity, digestibility, and heat stability. Bayer and KWS state that DMO has a documented history of safe consumption; is present at low levels in sugar beet root; lacks similarity to known allergens, toxins, or other biologically active proteins known to have adverse effects on humans; is degraded by the digestive enzymes pepsin and pancreatin; loses activity after heat treatment; and is not acutely toxic. Bayer and KWS conclude, based on the weight of evidence, that DMO from KWS20-1 sugar beet poses no meaningful risk to human health.

In a previous consultation, BNF 000125 (MON 87708 soybean), a series of *in vitro* DMO activity assays was conducted to determine whether DMO could catalyze reactions with endogenous substances in plants and produce unintended reaction products that would raise food safety concerns. The results showed that DMO lacked activity on endogenous soybean substances with structural similarity to dicamba. The version of DMO assessed in BNF 000125 is similar to the DMO from the source organism, with the exception of the presence of an N-terminal histidine (HIS) tag. The version of DMO expressed in KWS20-1 sugar beet lacks the N-terminal HIS tag but differs from the DMO from the source organism by the addition of one amino acid at position 2 and an N-terminal chloroplast transit peptide. To ensure these differences do not affect DMO's substrate specificity, Bayer and KWS assessed the specificity of the version of DMO from KWS20-1 sugar beet on *o*-anisic acid, the endogenous compound identified in BNF 000125 to be the most structurally similar to dicamba, and therefore considered the most likely to be an unintended substrate of DMO. Using an *in vitro* reaction, liquid chromatography, and mass spectrometry, Bayer and KWS found no evidence of DMO activity on *o*-anisic acid. Bayer and KWS further conducted a literature search and found no endogenous substance from sugar beet with structural similarity to dicamba (e.g., phenyl carboxylic acids containing methoxy moieties and chlorine moieties). Bayer and KWS conclude that DMO expressed in KWS20-1 sugar beet is unlikely to catalyze reactions with endogenous substances in sugar beet and produce unintended reaction products to an extent that would raise food safety questions.

[7] Klein, J. et. al, 1998. Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugar beets. Journal of Biotechnology 60(3):145-153.

Introduced Protein: phosphinothricin-N-acetyltransferase (PAT)

Intended trait	Tolerance to glufosinate
Source organism	*Streptomyces viridochromogenes*
Protein description	PAT protein expressed in KWS20-1 sugar beet is identical to the wild-type PAT from *Streptomyces viridochromogenes,* except for the removal of the first-position methionine during co-translation processing of the protein in sugar beet.
Intended function	PAT catalyzes the acetylation of glufosinate herbicide to produce non-herbicidal N-acetyl glufosinate.

Bayer and KWS used an ELISA to measure the level of PAT protein in KWS20-1 sugar beet root. The mean level of PAT in root was detected at a range from 0.125-0.25 μg/g dry weight. Bayer and KWS conclude that the concentration of PAT in sugar beet root tissues is very low and cite a publication documenting that refined sugar from sugar beets does not contain detectable levels of protein.

FDA previously evaluated PAT in consultations BNF 000135, BNF 000148 and BNF 000173. Bayer and KWS include information from previous consultations by reference. Bayer and KWS also refer to published safety assessments of PAT, which cite studies on acute oral toxicity, digestibility, and heat stability. Bayer and KWS state that PAT has a documented history of safe consumption; is present at low levels in sugar beet root; lacks similarity to known allergens, toxins, or other biologically active proteins known to have adverse effects on humans; is degraded by the digestive enzymes pepsin and pancreatin; loses activity after heat treatment; and is not acutely toxic. Bayer and KWS conclude, based on the weight of evidence, that PAT from KWS20-1 sugar beet poses no meaningful risk to human health.[8]

Introduced Protein: 5-enolpyruvylshikimate-3- phosphate synthase (CP4 EPSPS)

Intended trait	Tolerance to glyphosate
Source organism	*Agrobacterium* sp. strain CP4
Protein description	CP4 EPSPS expressed in KWS20-1 sugar beet is identical to wild type EPSPS from *Agrobacterium* sp. strain CP4, except for the addition of a chloroplast targeting sequence.
Intended function	CP4 EPSPS is functionally equivalent to endogenous EPSPS but has reduced affinity to glyphosate.

Bayer and KWS used an ELISA to measure the level of CP4 EPSPS protein in KWS20-1 sugar beet root. The mean level of CP4 EPSPS in root was detected at a range from 100-430 mg/g dry weight. Bayer and KWS conclude that the concentration of CP4 EPSPS in sugar beet root tissues is very low and cite a publication documenting that refined sugar from sugar beets does not contain detectable levels of protein.

FDA evaluated CP4 EPSPS in previous consultations, including BNF 000173. Bayer and KWS include information from previous consultations by reference. Bayer and KWS also refer to published safety assessments of CP4 EPSPS, which cite studies on acute oral toxicity, digestibility, and heat stability.

[8] Although PAT is not used as a plant-incorporated protectant (PIP) inert ingredient in KWS20-1 sugar beet, its safety is supported by an EPA exemption from the requirement of a tolerance for PAT in all food commodities when used as a PIP inert ingredient under 40 CFR 174.522.

Bayer and KWS state that CP4 EPSPS has a documented history of safe consumption; is present at low levels in sugar beet root; lacks similarity to known allergens, toxins, or other biologically active proteins known to have adverse effects on humans; is degraded by the digestive enzymes pepsin and pancreatin; loses activity after heat treatment; and is not acutely toxic. Bayer and KWS conclude, based on the weight of evidence, that CP4 EPSPS from KWS20-1 sugar beet poses no meaningful risk to human health.[9]

Intended Human Food Uses

Sugar beet is grown primarily for its root, which has a high sugar content. Unprocessed sugar beets are seldom used for human food; rather, sugar beet roots are processed into sugar and pulp. According to Bayer and KWS, refined sugar is the predominant use of sugar beet root while sugar beet fiber produced from sugar beet pulp has limited applications in human food in the United States.

Compositional Assessment

Bayer and KWS analyzed roots from KWS20-1 sugar beet, a near-isogenic sugar beet control variety, and multiple commercial reference varieties, grown in five locations representing typical agricultural regions for sugar beet production in the United States in 2020.

Bayer and KWS analyzed roots for the levels of nutrients including proximates (protein, total fat and ash), carbohydrate by calculation, sucrose, amino acids, fiber (crude fiber and pectin), and minerals (phosphorus, potassium and sodium). Statistical differences in the mean levels for lysine, proline, threonine, serine, total fat, ash, phosphorus, and potassium were observed between KWS20-1 and near-isogenic line control. However, the mean values of these eight components were within the ranges observed for the control variety and commercial reference varieties and within the ranges reported in the Agriculture & Food Systems Institute Crop Composition Database (AFSI-CCDB)[10]. Bayer and KWS concluded that the KWS20-1 sugar beet is compositionally comparable to near-isogenic control sugar beet and commercially available reference sugar beet varieties.

Conclusion

Based on the information provided by Bayer and KWS and other information available to HFP, we have no further questions at this time about the safety, nutrition, and regulatory compliance of human food from KWS20-1 sugar beet. We consider the consultation with Bayer and KWS on KWS20-1 sugar beet to be complete.

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[9] Although CP4 EPSPS is not used as a PIP inert ingredient in KWS20-1 sugar beet, its safety is supported by an EPA exemption from the requirement of a tolerance for CP4 EPSPS in all food commodities when used as a PIP inert ingredient under 40 CFR 174.523.

[10] In 2020, AFSI-CCDB had sugar beet compositional data available from 2008 crop year.