Biotechnology Notification File No. 000170
CFSAN Note to the File

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To: Administrative Record, BNF No. 000170

Subject: Wheat with transformation event IND-ØØ412-7 (IND-ØØ412-7 wheat)

Keywords: *Triticum aestivum* L., wheat, tolerance to environmental stress, *HaHB4* gene, transcription factor, HAHB4, *Helianthus annuus*, sunflower, herbicide tolerance, glufosinate ammonium, *bar* gene, phosphinothricin acetyltransferase, PAT, *Streptomyces hygroscopicus*, Bioceres Inc., HB4 wheat, OECD unique identifier IND-ØØ412-7

Summary

Bioceres Inc. (Bioceres) has completed a consultation with the Food and Drug Administration (FDA) on food derived from wheat event IND-ØØ412-7 with tolerances to environmental stress and glufosinate ammonium herbicides,[1] conferred through expression of an *HaHb4* gene from *Helianthus annuus* and the *bar* gene from *Streptomyces hygroscopicus*, respectively. This document summarizes Bioceres' conclusions and supporting data and information that FDA's Center for Food Safety and Applied Nutrition (CFSAN, 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.

Bioceres concludes:

- it has not introduced into human food a new protein or other substance that would require premarket approval as a food additive.
- human food from IND-ØØ412-7 wheat is comparable to and as safe as human food from other bread wheat varieties.

CFSAN evaluated data and information supporting these conclusions and considered whether IND-ØØ412-7 wheat raises other regulatory issues involving human food under the Federal Food Drug and Cosmetic Act. We have no further questions at this time about the safety, nutrition, and regulatory compliance of food from IND-ØØ412-7 wheat.

[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 & Cosmetic Act, EPA establishes tolerances (maximum legally permissible levels) of residues of pesticides in food.

Subject of the Consultation

Crop	Wheat
Designations	IND-ØØ412-7
Intended Trait	Tolerance to environmental stress
Intended Trait	Tolerance to glufosinate herbicides
Developer	Bioceres Inc.
Submission received	September 17, 2018
Amendments received	October 25, 2018; October 23 and December 22, 2020; and October 25, 2021
Intended use	Use in processed food products
Transformation plasmid	Plasmids *pIND4-HB4* and *pIND4-Bar*
Expression cassette 1	*HaHB4* expression cassette intended to confer increased tolerance to environmental stresses that can reduce crop yield; the *HaHB4* coding sequence is under the regulatory control of a *Zea mays Ubi-1* promoter construct
Expression cassette 2	*bar* expression cassette intended to confer tolerance to glufosinate herbicides; the *bar* coding sequence is under the regulatory control of a *Zea mays Ubi-1* promoter construct
Method for conferring genetic change	Particle bombardment-mediated co-transformation

Molecular Characterization

Confirmation of intended genetic change

Because the wheat genome is large, complex, and repetitive, Bioceres characterized the genetic change using a combination of techniques. Bioceres reported that the results of Southern blot analysis of IND-ØØ412-7 wheat genomic DNA indicated a complex insertion pattern consisting of multiple copies of both the *HaHB4* and *bar* coding sequences, with possible internal DNA rearrangements. Consequently, Bioceres undertook a genomic sequencing approach to determine the insertion structure. Diversity Arrays Technology (DArT) was used to identify the chromosome into which the transgenic DNA had integrated. The identified chromosome was then isolated using flow cytometry and the insertion and flanking genomic DNA sequences were determined using two complementary methods. The first method produced short sequencing reads with high coverage, which were then assembled using the second, longer read method.

Bioceres reported that the results are consistent with two complex DNA insertions in a single genetic locus on chromosome 2D: a long insert (approximately 47 kB) and a short insert (approximately 20 kB). Together, the DNA inserts contain elements from the intended *HaBH4*

and *bar* expression cassettes as well as unintended DNA vector elements derived from the transformation process.

With respect to the intended protein expression cassettes, IND-ØØ412-7 wheat contains three copies of the *HaHB4* cassette and eight copies of the *bar* cassette. However, only one of the *HaHB4* cassettes and three of the *bar* cassettes possess functional coding sequences: that is, a complete coding sequence with the necessary regulatory elements in the correct orientation for expression. The remaining copies have truncated coding sequences, and/or lack the necessary regulatory elements.

Bioceres cited several scientific publications showing that transformation of plants by particle bombardment can lead to complex transformation events. Bioceres stated that the data and information presented in its dossier demonstrate that the complexity of the DNA insertion locus does not impact the food safety or expected inheritance pattern.

Presence of unintended DNA vector elements

Bioceres reported that sequencing results indicated the presence of several unintended DNA vector elements, derived from the transformation process, in IND-ØØ412-7 wheat. These include complete and incomplete copies of the *bla* coding sequence and incomplete copies of the *gus* coding sequence from *Escherichia coli*; the *pBR322* replication origin; the 7S wheat globulin promoter (*prGbl-1)*; and the cauliflower mosaic virus transcription terminator *(T35S*). The *bla* gene originates from the *pIND4-HB4* and *pIND4-Bar* plasmid vector backbone sequences; copies of the *bla* gene present in IND-ØØ412-7 wheat do not possess regulatory elements for expression in wheat and are not positioned within the DNA insert near eukaryotic regulatory elements. Bioceres theorized that the *gus* coding sequence and the *prGbl-1* promoter and *T35S* transcription terminator – sequences that are not present in the *pIND4-HB4* and *pIND4-Bar* plasmids – are derived from a transformation efficiency plasmid used by the facility that prepared the event. Bioceres stated that all copies of the *gus* coding sequence present in IND-ØØ412-7 wheat are truncated and lack the appropriate regulatory elements for transcription.

Inheritance and stability

Bioceres examined the stability and inheritance of the two DNA inserts. In segregation studies, F2 progeny resulting from a cross between IND-ØØ412-7 wheat and a commercial wheat variety were analyzed using PCR primers designed to detect insert elements (e.g., *HaHB4*, *bar, bla*, and *gus*) and the four insert-to-genome junction sequences. Analyses of these F2 individuals showed either the presence or absence of all primer-targeted elements, indicating co-segregation of the two DNA inserts as a single, stable locus. Bioceres reported that Chi square analyses of the observed results show inheritance of the locus according to Mendelian principles. Bioceres verified the stability of the DNA insertions through PCR-based analyses of three generations of IND-ØØ412-7 wheat (T5, T6, and T7) using the same set of primers utilized for the segregation analysis. Bioceres reported that all samples were positive for the complete set of insert elements and concludes that the insertion locus is stable.

Open reading frame analysis

Bioceres used bioinformatic analyses to assess whether new ORFs were generated as a result of the DNA insertions and, if so, whether any raised toxicity or allergenicity concerns relevant to human food.

Specifically, Bioceres analyzed the nucleotide sequences of the two DNA inserts and 200 base pairs of each of the four genome flanking sequences in IND-ØØ412-7 wheat for the presence of start codons with the potential to produce peptides of greater than 100 amino acids.[2] The putative peptides were then compared to proteins in the National Center for Biotechnology Information (NCBI) database of non-redundant protein sequences. According to Bioceres' results, 44 of the 67 putative peptides share significant homology with proteins from the transformation plasmids (HAHB4, PAT, β-lactamase, and β-glucuronidase) while the remaining 23 do not share significant sequence homology with known proteins.[3] Bioceres determined that none of the putative peptides share significant homology to known toxins or allergens by comparing these to the sequences of known toxins in the NCBI non-redundant protein database and the Animal Toxins Data Base (ATDB; He et al., 2008) and known allergens in the Food Allergy Research and Resource Program (FARRP) and Structural Database of Allergenic Proteins databases (SDAP; Ivanciuc et al., 2002, 2003).[4]

Bioceres also conducted bioinformatic analyses using a more conservative approach, analyzing putative peptides of 25 or more amino acids. A total of 318 putative peptides were found. Bioceres compared the putative peptides to sequences in several public databases, including the NCBI non-redundant protein and FARRP allergen databases. Consistent with the results of the preceding analyses, Bioceres reported that the putative peptides do not share significant sequence homology to known toxins or allergens.

Bioceres considered the potential for expression of the *bla* and *gus* coding sequences present in the DNA inserts and concluded these are unlikely to result in the transcription of either β-lactamase or β-glucuronidase because the coding sequences present in IND-ØØ412-7 wheat lack intact regulatory elements for expression in plants. Nevertheless, Bioceres summarized available information on the presence and safety of the *bla* and *gus* genes, and their expression products, in the environment and in food. Bioceres concludes that the *bla* and *gus* sequences present in IND-ØØ412-7 wheat do not raise food safety concerns.

Bioceres concludes that the results of its open reading frame analyses do not raise food safety concerns.

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[2] Bioceres summarized published literature supporting the prediction of open reading frames based on the presence of a canonical AUG start codon, a stop codon, and a minimum codon length of 100.
[3] Significant homology (E score < 10^{-5}) was assessed using the BLASTP algorithm.
[4] Significant homology to known allergens was defined as equal to or greater than 35% homology over a sliding 80 amino acid window or 100% identity across 8 contiguous amino acids.

Introduced Protein: HAHB4

Intended trait	Tolerance to environmental stress
Source organism	*Helianthus annuus* (sunflower)
Protein description	HAHB4, an HD-Zip I transcription factor
Intended function	Regulates transcription of genes involved in plant response to environmental stresses (such as drought, salinity, darkness, insect feeding, and chemical exposure)

HAHB4 safety assessment

To measure the expression levels of HAHB4 in grain from IND-ØØ412-7 wheat, Bioceres used a targeted Liquid Chromatography Mass Spectrometry (LC-MS) method with a validated detection limit of approximately 0.01 ng/g fresh weight (FW) and a lower limit of quantitation of 30 ng/g FW. Bioceres reported that HAHB4 was not detectable in mature grain samples from plants grown under field trial conditions.[5] Bioceres concluded that HAHB4 expression level is extremely low in mature grain, the part of the wheat plant used in human food.

Bioceres reported that the deduced amino acid sequence of the HAHB4 expressed in IND-ØØ412-7 wheat is identical to the HAHB4 proteins that are the subjects of FDA New Protein Consultation (NPC) 16 and Biotechnology Notification File (BNF) 155. The amino acid sequences of these differ from the amino acid sequence of the *Helianthus annuus* HAHB4 as deduced from its nucleotide sequence in the National Center for Biotechnology Information's GenBank (Accession AF339748.1). The differences include a deletion of 4 amino acids and 3 separate single amino acid substitutions. Bioceres stated that these differences are either conservative and/or do not have significant effects on the protein structure.[6] Bioceres concludes that it does not expect a negative impact to the protein food safety properties as a result of these differences.

Bioceres summarized the weight of evidence on which it assessed the safety of HAHB4 in IND-ØØ412-7 wheat, emphasizing its low expression level in wheat grain and its equivalence to the HAHB4 proteins evaluated by FDA in NPC 16 and BNF 155. Bioceres incorporated data and information about HAHB4, including its source and function as an HD-Zip I transcription factor associated with plant stress-response pathway, by reference to BNF 155. Bioceres discussed the results of (1) bioinformatic comparisons of sequence homology between the HAHB4 and known allergenic and toxic proteins, (2) analyses for glycosylation potential, and (3) assays for physical-chemical properties (e.g., digestibility in simulated gastric fluid and thermal stability). As reported in BNF 155, HAHB4 does not share significant sequence similarity to known toxins [3]

[5] Bioceres reported HAHB4 was detectable in IND-ØØ412-7 wheat seedlings collected from growth chamber experiments designed to elicit HAHB4 expression through exposure to osmotic stressors. The highest measured value was 0.0018 ng/g FW.
[6] As assessed using the Protein Variation Effect Analyzer (PROVEAN) algorithm (http: provean.jcvi.org).

and allergens,[4] and it is unlikely to be glycosylated as it lacks signal peptide and glycosylation sites. It is heat stable (90°C for up to 60 minutes) but sensitive to digestion by pepsin (digestion time <30 seconds). Bioceres concludes that although the HAHB4 is heat stable, the weight of the evidence supports the conclusion that the HAHB4 is unlikely to cause an allergic reaction or be toxic to humans and therefore is safe for human consumption.

Introduced Protein: PAT

Intended trait	Tolerance to glufosinate ammonium herbicides
Source organism	*Streptomyces hygroscopicus*
Intended function	PAT catalyzes the acetylation of glufosinate ammonium herbicide

PAT safety assessment

Bioceres measured the levels of PAT in mature grain samples obtained from field trials conducted during the 2013 growing season. The samples were analyzed using a commercially available ELISA kit and results were compared to a standard curve of recombinant PAT. While no PAT was detected in the parent variety Cadenza, Bioceres reported that average PAT levels in IND-ØØ412-7 wheat grain across sites ranged from 1.8 to 3.8 µg/g FW. Bioceres noted that these levels are within the range reported in the literature for phosphinothricin-tolerant genetically engineered plants that have obtained regulatory approvals.[7]

Bioceres stated that the amino acid sequence of PAT expressed in IND-ØØ412-7 wheat, as deduced from the transformation construct and nucleotide sequencing, is the same as the sequence of the PAT protein present in HB4 soybean, the new plant variety evaluated in FDA Biotechnology Consultation BNF 155. Given this equivalence, Bioceres assessed the safety of IND-ØØ412-7 wheat-derived PAT in human food based on publicly available data and information in published peer-reviewed scientific literature on PAT proteins and in reports of previously evaluated plant varieties. Bioceres cited the results of bioinformatic assessments, assays measuring sensitivity heat and degradation by pepsin and pancreatin, and oral toxicity studies in rodents.[8] Based on the weight of publicly available evidence, Bioceres concludes PAT proteins generally, and by extension IND-ØØ412-7 wheat-expressed PAT, have been proven to be safe, have not changed the levels of natural constituents of food from plants, and have not shown potential toxicity, allergenicity or any nutritional quality concerns.[9]

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[7] International Life Sciences Institute (2016). A Review of the Food and Feed Safety of the PAT protein. ILSI Research Foundation, Washington D.C. USA

[8] Hérouet et al., (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. *Regulatory Toxicology and Pharmacology* **41**: 134-149.

[9] Although PAT is not used as a plant-incorporated protectant (PIP) inert ingredient in IND-ØØ412-7 wheat, 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.

Human Food Nutritional Assessment

To ensure the absence of unintended changes in components relevant to the safety or nutrition of human food, Bioceres analyzed mature grain samples collected from field trials conducted in Argentina during two growing seasons (2012 and 2015).[10] Samples from IND-ØØ412-7 wheat, the parent line Cadenza (control), and several commercial (reference) varieties were analyzed for key nutrients, including proximates (moisture, protein, fat, ash, and carbohydrates), starch, and dietary fiber, as well as minerals, fatty acids, amino acids, and vitamins relevant to the use of wheat in human food.[11] Bioceres also measured phytic acid and gliadin.

Bioceres reported that no differences were found between IND-ØØ412-7 wheat and the control for the majority of the components in the combined site analysis where no interaction between location and genotype was seen. Statistically significant differences were observed in the 2012 field trial for zinc, folic acid, serine, and threonine and in the 2015 field trial for protein, zinc, and leucine. Bioceres explained the magnitude of these differences was small and levels of these components in IND-ØØ412-7 wheat, with the exception of serine, were similar to values obtained for the reference varieties, reported in the scientific literature, or both. The level of serine in IND-ØØ412-7 wheat was slightly higher than in the control and reference lines but below the cited literature range, suggesting a regional effect. Bioceres' analyses showed an interaction between location and genotype for several components. While some significant differences between IND-ØØ412-7 wheat and the control were seen at one or more individual sites, most of the IND-ØØ412-7 wheat values were within the range obtain for the reference varieties, literature, or both. Bioceres concludes that the differences observed at individual sites are not biologically significant and that the composition of IND-ØØ412-7 wheat is similar to the control and conventional wheat.

Conclusions

Based on the information provided by Bioceres and other information available to CFSAN, we have no further questions at this time about the safety, nutrition, and regulatory compliance of human food from IND-ØØ412-7 wheat. We consider the consultation with Bioceres on IND-ØØ412-7 wheat to be complete.

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[10] Bioceres confirmed that the field trials and compositional data included in its dossier are the same as those subsequently reported in Ayala et al., (2019). Compositional equivalence of event IND-ØØ412-7 to non-transgenic wheat. *Transgenic Research* **28**:165–176.

[11] Compositional analytes were selected based on the OECD Consensus document on compositional considerations for new varieties of bread wheat (*Triticum aestivum*): key food and feed nutrients, anti-nutrients and toxicants. ENV/JM/MONO (2003).