Biotechnology Notification File No. 000192
CFSAN Note to the File

Date: September 13, 2024

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To: Administrative Record, BNF No. 000192

Subject: Apple with transformation event PG451 (PG451 Apple)

Keywords: Apple, *Malus x domestica*, Arctic apple, Pacific Gala (PG) cultivar, PG451, RNA interference (RNAi), polyphenol oxidase (PPO), reduced polyphenol oxidases, enzymatic browning, *Neomycin phosphotransferase type* II (*npt*II), *Escherichia coli* transposon Tn5, Okanagan Specialty Fruits Inc., OECD unique identifier OKA-PGØØ4-1

Summary

Okanagan Specialty Fruits Inc. (OSF) has completed a consultation with the Food and Drug Administration (FDA) on food derived from PG451 apple with reduced levels of polyphenol oxidases through RNA interference to impart resistance to enzymatic browning and with the expression of the NPTII protein as a selectable marker. This document summarizes OSF's 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.

Based on the safety and nutritional assessment OSF has conducted, it is our understanding that OSF concludes:

- it has not introduced into human food a new protein or other substance that would require premarket approval as a food additive, and
- human food from PG451 apple is comparable to and as safe as human food from other apple varieties.

CFSAN evaluated data and information supporting these conclusions and considered whether PG451 apple raises other regulatory issues involving human food within FDA's authority under the Federal Food, Drug, and Cosmetic Act (FD&C Act). We have no further questions at this time about the safety, nutrition, and regulatory compliance of human food from PG451 apple.

Subject of the Consultation

Crop	Apple
Designation	PG451
Intended trait	Reduced levels of polyphenol oxidases to impart resistance to enzymatic browning
Developer	Okanagan Specialty Fruits, Inc.
Submission received	May 25, 2022
Amendment received	February 5, 2024
Intended use	General use in human food
Transformation plasmid	GEN-03
Expression cassette 1	A chimeric RNAi sequence comprising fragments of four apple PPO genes (*PPO2*, *GPO3*, *APO5* and *pSR7*) to suppress the entire apple PPO gene family and to induce a non-browning phenotype in the apple fruit.
Expression cassette 2	*nptII* gene encoding a mutant neomycin phosphotransferase type II (NPTII) [1] from *Escherichia coli* Tn5 to provide resistance to kanamycin.
Method for conferring genetic change	*Agrobacterium*-mediated transformation

Molecular Characterization

Confirmation of intended genetic change

OSF transformed the Pacific Gala cultivar (PG) with the vector GEN-03 using *Agrobacterium*-mediated transformation to develop the PG451 apple.

OSF characterized the DNA insertions in PG451 apple using whole genome sequencing and bioinformatics analysis. OSF determined its average sequence coverage to be 24.3x. To identify junction sequences, OSF used next generation sequencing and a junction mapping process to identify the sequences that are matching both the GEN-03 plasmid vector and the apple reference genome.

OSF identified two apple/vector junctions co-located on Chromosome 10 (CHR10) and two apple/vector junctions co-located on Chromosome 17 (CHR17) suggesting two T-DNA insertion sites. The insertion in one chromosome contains three intact copies of T-DNA, with an inverted copy between the other two copies. There is also a partial vector backbone sequence between the

[1] OSF referred to an NPTII-mutant in this submission. The NPTII mutant has a single amino acid substitution with reduced phosphotransferase activity compared to the wild type. OSF stated that they have no safety concerns regarding the NPTII mutant. OSF noted the NTPII mutant gene sequence used in BNF 192 is the same as those in BNF 132 and BNF 154.

first T-DNA copy and the second, inverted T-DNA copy. The insertion in the other chromosome contains two intact copies of T-DNA separated by a complete copy of the vector backbone sequence. OSF identified two internal vector-to-vector junctions which cannot be definitively assigned to either CHR10 or CHR17, and which OSF explains does not affect the conclusion of the safety and nutritional assessment of PG451 apple. OSF explains that the genetic elements in the vector backbone are unlikely to pose a safety concern as they lack the essential functional sequences for expression in plant cells.

Inheritance and stability

Commercial apples are vegetatively propagated and do not undergo processes associated with genetic variation such as meiosis, recombination, or segregation. Therefore, genotypes and phenotypes of apple cultivars are expected to remain relatively stable. OSF developed PG451 apple in 2011, propagated this cultivar vegetatively through multiple generations in tissue culture, and grafted it onto rootstocks in 2016. Molecular characterization, phenotypic, nutritional, and compositional analyses were performed on samples collected from a field trial in 2021. In addition, PCR analysis of leaf samples collected in 2021 confirmed the presence of the *nptII* selectable marker gene. OSF therefore concludes that the results from these analyses are consistent with the long-term stability of the insertions and of the non-browning trait in PG451 apple.

Open reading frame analysis

OSF used bioinformatic analysis to assess deduced translation products of putative open reading frames (ORFs) generated by the insertions and in the genomic regions flanking each insertion for homology to known allergens or toxins. The entire GEN-03 vector sequence and the unique junction sequences in PG451 were translated in all six reading frames and deduced amino acid sequences of greater than 29 amino acids were chosen for further analysis. OSF used the Food Allergy Research and Resource Program (FARRP) allergen protein database[2] (Version 20) for comparison of the deduced amino acid sequences to known allergens. OSF reports that none of the identified putative ORFs met the threshold[3] to generate a match with a known allergen. To assess the potential toxicity of the inserted sequences, OSF ran the putative ORFs through the blastp tool against the National Center for Biotechnology Information (NCBI) non-redundant protein sequence database.[4] The putative ORFs that generated hits were individually aligned with the NCBI online blastp tool and the results searched for presence of the words “toxin” or “toxic.” OSF reports that no instances of “toxin” or “toxic” were found in the alignment results generated. OSF therefore concludes that none of the putative peptides would be sufficiently similar to known allergens or toxins.

Introduced Protein: Neomycin phosphotransferase II (NPTII)

Intended trait	Resistance to kanamycin, for use as a selectable marker during transformation

[2] AllergenOnline (http://www.allergenonline.org/databasehelp.shtml)
[3] 50% identity over the length of the entire amino acid sequence with an e-value of 10^-4 and 35% identity or greater over 80 amino acids in a sliding window.
[4] NIH-NLM-NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Source organism	*Escherichia coli* transposon Tn5
Protein description	Aminoglycoside phosphotransferase
Intended function	Phosphorylation of aminoglycoside antibiotics

OSF estimated NPTII protein levels in mature PG451 apple fruit using a semi-quantitative NptII ELISA approach. OSF analyzed six apples of PG451 and six apples of Pacific Gala (PG control) harvested in 2021. The levels of NPTII protein in all samples tested were below the limit of quantitation (10 ng/g fresh weight of apple). OSF used the same vector GRN-03 to generate PG451 apple as GD743 and GS784 apple cultivars (subjects of BNF No. 000132) and NF872 apple cultivars (subject of BNF No. 000154) and discussed the safety of NPTII protein in these two previous BNF submissions. OSF concludes that no new protein that may be toxic or allergenic is expressed in PG451.

Intended Human Food Uses

PG451 apple will be used as direct replacement of conventional Gala apples where the non-browning trait is considered desirable.

Characterization of the Intended Trait

OSF developed PG451 apple to be resistant to enzymatic browning by suppressing expression of four PPO genes. OSF measured PPO activity in mature fruit and compared levels in PG451 apple to the parent apple cultivar Pacific Gala. PPO activity was completely suppressed in mature fruits of PG451 apple relative to the PG control.

Bruising experiments conducted on mature apples showed a visibly significant reduction in the browning response in PG451 apple compared to the PG control. OSF states that other forms of mechanical damage, such as slicing or juicing, have yielded the same non-browning phenotype for PG451. OSF concludes that the targeted PPO genes are functionally suppressed in PG451 apple, resulting in the non-browning phenotype.

Human Food Nutritional Assessment

The intended trait in PG451 apple was not expected to alter levels of key nutrients, anti-nutrients, or toxicants. To assess potential unintended changes in composition relevant to safety or nutrition, OSF analyzed fruit from PG451 and PG control apple trees planted in New York State in 2016 and Washington State in 2016 and 2019. At each location, mature fruit was harvested and pooled by variety. OSF measured proximates (fat, protein, moisture, ash, calculated carbohydrates, and calories), sugars, total dietary fiber, potassium, vitamin C, and phenolics. OSF analyzed six samples each for the PG451 and PG control apples. Data were

pooled across locations for each cultivar. OSF compared its results to values from the publicly available USDA database[5] for apple as well as for Gala apple specifically.

OSF found no significant differences in the levels of most nutritional components in PG451 and PG control apples, except for potassium that appeared higher in PG451. Upon further examination of the data by sample and location, OSF discovered that the potassium levels in both PG451 and PG control from Washington State were much higher than those from New York State. This was attributed to soil condition, weather, and harvest time differences between the two locations. OSF notes that vitamin C and phenolics in both PG451 and PG control were below the limit of detection. OSF concludes that PG451 is nutritionally equivalent to its parent variety and as safe as its parent variety.

Labeling considerations

It is a producer's or distributor's responsibility to ensure that labeling of the foods it markets derived from PG451 apple meets applicable legal requirements, including disclosure of any material differences (for example, differences in function, composition, and nutritional or safety profiles) in the food as compared to its conventional counterpart. It is our understanding that PG451 apple may be used in various food applications. Depending on the particular food application, the non-browning aspect of the apples may be considered a material fact requiring disclosure under Sections 201(n) and 403(a)(1) of the Federal Food, Drug, and Cosmetic Act [21 U.S.C. § 321(n) and 343(a)(1)]. Companies marketing food from PG451 apple are advised to consult with CFSAN's Office of Nutrition and Food Labeling, Division of Food Labeling and Standards, to discuss any required or voluntary labeling including statements relating to attributes of PG451 apple and products produced from it. Failure to do so may result in misbranding of products produced from PG451 apple within the meaning of Sections 201(s) and 403(a)(1) of the FD&C Act.

Conclusion

Based on the information provided by OSF 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 PG451 apple. We consider the consultation with OSF on PG451 apple to be complete.

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[5] For proximates: USDA National Nutrient Database for Standard Reference, Release 28 slightly revised Sept 23, 2021. For Phenolics: USDA Database for the Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, Release 2, May 2010.