Biotechnology Notification File No. 000192
CVM Note to the File

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From: Ramavati Pal, Ph.D.

To: Administrative Record, BNF No. 000192

Subject: Event PG451 apple

Keywords: Apple, *Malus x domestica*, Arctic® apple, Pacific Gala (PG), PG451, Ribonucleic acid interference (RNAi), Polyphenol oxidase (PPO), Resistance to enzymatic browning, *Neomycin phospho-transferase type* II (*nptII*) gene from *Escherichia coli* transposon Tn5, OECD unique identifier OKA-PGØØ4-1, Okanagan Specialty Fruits, Inc.

Purpose

This document summarizes the Food and Drug Administration (FDA) Center for Veterinary Medicine's (CVM, we) evaluation of biotechnology notification file (BNF) number 000192. Okanagan Specialty Fruits, Inc. (OSF) submitted a safety and nutritional assessment for a biotechnology derived apple with reduced levels of polyphenol oxidases, transformation event PG451 (hereafter referred to as PG451 apple). CVM evaluated the information in OSF's submission and additional information afterwards to ensure that regulatory and safety issues regarding animal food derived from PG451 apple have been resolved prior to commercial distribution. FDA's Center for Food Safety and Applied Nutrition summarizes its evaluation of PG451 apple for use in human food in a separate document.

In CVM's evaluation, we considered all of the information provided by OSF 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

One of intended effects of the modifications in PG451 apple is to reduce enzymatic browning. To confer this trait, OSF introduced a chimeric construct which is designed to simultaneously suppress expression of four members of the apple PPO gene family through the RNA interference (RNAi). The second intended effect is to express the *neomycin phospho-transferase type II* (*nptII*)[1] gene from *Escherichia coli* transposon *Tn5* that encodes for NPTII protein which serves as a selectable marker.

[1] Also known as aminoglycoside 3'-phospho-transferase II.

Regulatory Considerations

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

Agrobacterium-mediated transformation with the GEN-03 vector[2] was used to insert the transfer DNA (T-DNA) into the genome of the apple cultivar PG. The T-DNA in GEN-03 vector is delineated by left and right border sequences. The T-DNA contains two cassettes: a chimeric PPO RNAi construct consisting of 394, 457, 457 and 453 base-pair (bp) regions of four apple PPO genes (*PPO2, GPO3, APO5,* and *pSR7*, respectively) placed in the sense orientation and a *nptII* gene from *Escherichia coli* transposon Tn5. Well characterized promoter and terminator sequences were used in these cassettes and are described in detail in FDA's memorandum of BNF No. 000132.

Characteristics of the Introduced DNA

OSF isolated genomic DNA from tissue culture leaf samples of PG451 apple and conducted whole genome sequencing, followed by bioinformatics analysis. ThePG451 apple genome trimmed reads were mapped to the HFTH1 apple reference genome. OSF determined its average genome coverage to be 24.3x. To detect T-DNA insertions and identify junction sequences[3] matching both GEN-03 plasmid vector and the apple genome, OSF mapped apple genome reads to GEN-03 plasmid vector and to various apple reference genomes (HFTH1, GDDH13, v3.0.a1 and v1.0 combined haplotypes)[4].

OSF identified four apple/vector junction sequences: two apple/vector junction sequences co-located in chromosome 10 and two apple/vector junctions co-located in chromosome 17 suggesting two T-DNA insertions. The arrangement of vector sequences in the junctions indicated that both insertions were complex, comprising multiple vector fragments. The insertion in one chromosome contained three fragments of GEN-03 vector including three intact copies of the T-DNA, a partial copy of the vector backbone and two structural rearrangements. The insertion in other chromosome contained two fragments of Gen-03 vector including two intact copies of the T-DNA and a complete copy of the vector backbone. OSF states that the insertions identified internal junction sequences, each representing a junction between vector sequence fragments within an insertion. OSF states that the internal junctions cannot be definitively assigned to either

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[2] GEN-03 is based on the binary plasmid pBINPLUS which is based on the widely used binary plasmid BIN19.

[3] Junction sequence refers to the sequence between the introduced DNA and the native genomic DNA sequence.

[4] Apple reference genomes are available at the Genome Database for Rosaceae (https://www.rosaceae.org/)

CHR10 or CHR17[5]. Both the T-DNA insertions contained *nptII*-mutant gene[6].

Stability and Inheritance of the Introduced DNA

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 varieties are expected to remain relatively stable. OSF developed PG451 apple in 2011, propagated the variety through multiple generations of tissue culture, and grafted it onto rootstocks in 2016. Molecular characterization, phenotypic analysis, and compositional analysis were performed on samples collected in 2021. PCR analysis confirmed the presence of the *nptII* selectable marker gene in leaf samples collected in 2021. These results are consistent with the long-term stability of the insertions and of the non-browning trait.[7]

OSF performed bioinformatics analyses using the nucleotide sequences obtained for the inserted DNA and their corresponding flanking genomic junction sequences to determine whether insertion of the introduced DNA created any potential open reading frames (ORFs) that could encode for putative polypeptides. OSF reports that none of the putative polypeptides had alignments with proteins in its toxin database[8].

Protein Safety

OSF affirms that the introduction of the PPO RNAi construct does not result in any new protein production in event PG451. Rather, the introduction of the PPO RNAi construct results in a reduction in the production of endogenous PPO proteins. OSF states that the sole new protein expressed in event PG451 is the enzyme NPTII, encoded by the *nptII* gene. The NPTII protein confers resistance to the antibiotic kanamycin and was used as a selectable marker during the transformation of the parental apple varieties.

OSF estimated NPTII protein levels in mature fruit using a semi-quantitative enzyme-linked immunosorbent assay (ELISA) approach. OSF analyzed six fruits obtained from PG451 apple, and six fruits obtained from untransformed control (PG) harvested in 2021. Levels of NPTII in all samples were below the limit of quantitation, which was 10 ng/g fresh weight. OSF notes that NPTII is the only introduced protein in PG451 apple

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[5] OSF states that "since the chromosomes 10 and 17 have inserts that both begin around the transgene RB and end around the transgene LB, the internal vector-vector junctions cannot be anchored to either chromosome as the reads are not long enough to cover both a vector-vector junction and a vector-chromosome junction. The vector-vector junctions and direction of the elements do provide a logical order that the transgene copies (and backbone) need to be in so that they may all be accounted for". The exact chromosome location of each insertion, how each insertion begins and ends, and all elements of the vector that are within are known. However, the piece of information that is missing is which middle goes with which, but because the beginning and end are the same, OSF states that the pairing is inconsequential.

[6] 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.

[7] To confirm the stability of non-browning trait, OSF measured enzymatic browning that occurs in response to mechanical bruising in mature fruit of PG451 relative to its control cultivar PG using Minolta Chroma Meter CR-400. A subset of trees from an PG451 propagation block was screened.

[8] National Center for Biotechnology Information (NCBI) non-redundant protein database using Geneious.

and that the NPTII protein does not accumulate to detectable levels in mature apple fruit. Further, OSF notes that FDA has previously evaluated the safety of the use of the NPTII protein in the development of other GE crops, including cotton, oilseed rape and tomatoes for food and feed purposes (21 CFR 173.170 and 21 CFR 573.130 respectively). Therefore, no new protein that may be toxic is expressed in PG451 apple.

Evidence for lower PPO activity in tissues of PG451 apple

OSF used a functional assay to directly monitor PPO activity in apple tissues (tissue culture leaves, greenhouse leaves and mature fruit). OSF calculated levels of suppression of PPO activity for the tissue culture leaves 90 percent, for greenhouse leaves 74 percent and for mature fruit 100 percent in PG451 apple compared to PG controls. Suppression of PPO activity was found in fruit and leaf grown under different conditions. OSF concluded that these data support the conclusion that the targeted PPO genes are functionally suppressed in PG451 apple.

Enzymatic Oxidative Browning versus Spoilage and Microbial Degradation[9]

OSF notes that the enzymatic oxidative browning that normally occurs in apples takes place within minutes and is distinct from the spoilage process, which happens later, over a protracted time frame and is driven by bacteria and molds. Consequently, PG451 apples will spoil like other apples and the absence of enzymatic browning will make such degradation easily apparent.

Animal Food Use

Gala is one of the top seven apple varieties grown in the United States. Byproducts of processing, such as pomace left over from juice production, may be fed to animals[10].

Composition

Scope of Analysis

OSF analyzed the nutrient composition of mature fruit from PG451 apple and from PG control.

Study Design

OSF conducted two field trials: one in 2004 in Washington and one in 2005 in New York. The trees were planted on Malling 9 rootstocks. There were 14 PG451 trees and 14 PG control trees in New York field trial and total 31 PG451 trees and 11 PG control trees in Washington field trial. OSF harvested fruit from plantings of PG451 apple and PG control in both the field sites. For each variety and from each site, OSF analyzed six samples, each sample derived from one randomly selected apple from the planting. OSF measured crude fat, crude protein, moisture, ash, carbohydrates by calculation, calories, sugars, total dietary fiber, potassium, vitamin C, and phenolics. OSF pooled data across locations for each variety and presented the means, standard deviations, and ranges for

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[9] OSF addressed enzymatic oxidative browning versus spoilage and microbial degradation in BNF No. 000132

[10] Incorporated by reference in BNF No. 000132

each component. OSF compared its results to values from publicly available USDA databases[11] for apple as well as for Gala apple specifically.

Results of Analyses

OSF found no differences that would significantly affect nutrition between levels of components in PG451 apple and PG control. The values of fat (0.1%) and vitamin C (0.2mg/100g) were below the detection limit and phenolics were not detected in both PG451 and PG control. OSF states that levels of potassium appeared to be significantly higher in PG451 apple. However, within a field trial PG451 and PG have very similar levels of potassium and the discrepancy is rather between the two field trials. OSF concludes that PG451 apple is nutritionally equivalent to its parental variety.

Summary of Compositional Analyses

OSF highlights that the genetic modification does not meaningfully affect nutrient composition and nutritional value of fruit derived from PG451 apple. OSF concludes that PG451 apple is comparable to Gala apples that are currently used in animal food in the United States.

Conclusion

FDA evaluated OSF's submissions to determine whether PG451 apple raises any safety or regulatory issues with respect to its uses in animal food. Based on the information provided by the company and other information available to the agency, FDA did not identify any safety or regulatory issues under the FD&C Act that would require further evaluation at this time.

OSF has concluded that its non-browning apple variety, PG451 apple, and animal food derived from it are as safe as and are not materially different in composition or any other relevant parameter from other apple varieties now grown, marketed, and consumed. At this time, based on OSF's data and information, CVM considers OSF's consultation on PG451 apple for use in animal food to be complete.

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[11] For proximates-USDA National Nutrient Database for Standard Reference, Release 28 slightly revised Sept 23, 2021, Statistics Reports: Gala, raw, with skin (NDB09503), Apples, raw, with skin (NDB09003). For Phenolics- USDA Database for the Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, Release 2, May 2010. Gala, raw, with skin (NDB97067), Apples, raw, with skin (NDB09003).