U.S. FOOD & DRUG ADMINISTRATION
CENTER FOR VETERINARY MEDICINE

Biotechnology Notification File No. 000174
Center for Veterinary Medicine Note to the File

Date: August 13, 2021

From: Rial Christensen, Ph.D.

To: Administrative Record, BNF No. 000174

Subject: Event Z6 Potato

Keywords: Potato, *Solanum tuberosum* subsp. *tuberosum*, Retransformation of V11 variety (lower asparagine, lower reducing sugars, and reduced black spot), Ribonucleic acid interference, RNAi, Silencing of *VInv* gene, Vacuolar invertase, Lower reducing sugars, *Rpi-vnt1* gene, *Solanum venturii*, Vnt1 protein, Late blight resistance, *Phytophthora infestans*, OECD identifier SPS-000Z6-5, J.R. Simplot Company

Purpose

This document summarizes the Food and Drug Administration (FDA) Center for Veterinary Medicine's (CVM, we) evaluation of biotechnology notification file (BNF) number 000174. The J.R. Simplot Company (Simplot) submitted a safety and nutritional assessment for a genetically engineered (GE) potato, transformation event Z6 (hereafter referred to as Z6 potato) and additional information afterwards. We evaluated the information in Simplot's submissions to ensure that regulatory and safety issues regarding uses of Z6 potato in animal food have been resolved prior to commercial distribution. FDA's Center for Food Safety and Applied Nutrition summarizes its evaluation of uses of Z6 potato in human food in a separate document.

In CVM's evaluation, we considered all of the information provided by Simplot as well as publicly available information and information in the agency's files. Here we discuss the outcome of the consultation for use of Z6 potato in animal food, but do not intend to restate the information provided in the final consultation in its entirety.

Intended Effects

Simplot used potato variety event V11 (hereafter referred to as V11 potato)[1] to generate Z6 potato. The V11 potato was modified to reduce free asparagine levels, lower reducing sugars, and reduce black spot. One of the intended effects of the modification in Z6 potato is to further reduce the levels of reducing sugars. Simplot introduced DNA sequences containing inverted repeat segments of the *vacuolar invertase* gene (*VInv*), which produces double-stranded RNAs (dsRNAs) to reduce the RNA transcript levels of

[1] Development and safety of V11 potato was described in BNF No. 000152.

VInv.[2] Vacuolar invertase participates in converting sucrose to its component reducing sugars. Another intended effect is to confer late blight resistance. Simplot introduced the *Rpi-vnt1* gene from *Solanum venturii*, a wild potato species that grows in South America. The *Rpi-vnt1* gene encodes the resistance protein (R-protein) Vnt1, which confers resistance to late blight caused by *Phytophthora infestans*.

Regulatory Considerations

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

The Environmental Protection Agency (EPA) defines a plant-incorporated protectant (PIP) as "a pesticidal substance that is intended to be produced and used in a living plant, or the produce thereof, and the genetic material necessary for the production of such a pesticidal substance," including "any inert ingredient contained in the plant, or produce thereof" (40 CFR 174.3). EPA regulates PIPs under the FD&C Act and the Federal Insecticide, Fungicide, and Rodenticide Act. Under EPA regulations, the DNA sequences within the *Rpi-vnt1* expression cassette and its expression products in Z6 potato are considered pesticidal substances. Therefore, these substances fall under the regulatory purview of EPA.

Genetic Modification and Characterization

Introduced DNA and Transformation Method

Simplot used V11 potato to generate Z6 potato. The V11 potato was derived from the Snowden potato variety, which is primarily used in the production of potato chips. The V11 potato was transformed with the pSIM1278 plasmid[3], to reduce the levels of asparagine synthetase (*Asn1*), glucan water dikinase R1 (*R1*), phosphorylase L (*PhL*), and polyphenol oxidase (*Ppo5*) ribonucleic acid (RNA) transcripts.

Simplot transformed four to six millimeter internode segments of V11 potato with plasmid pSIM1678 using disarmed *Agrobacterium tumefaciens*-mediated transformation.[4] Previously, Simplot described that the transfer-DNA (T-DNA) region within this plasmid contained two expression cassettes between the left and right border sequences.[5] The first expression cassette contains the coding sequence of the *Rpi-vnt1* gene from *S. venturii*, as well as its native promoter and terminator sequences. The second expression cassette contains a partial sequence of the *VInv* gene from *S. tuberosum* var. *ranger russet*, followed by a spacer, which is then followed in succession by the complementary sequence of the partial *VInv* sequence in reverse orientation. The cassette is preceded by a copy of the *ADP-glucose pyrophosphorylase* promoter and

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[2] Hannon, G.J. 2002. RNA interference. Nature 418: 244-251.
[3] The pSIM1278 plasmid was first described in BNF No. 000146.
[4] The transformation method was initially described in BNF No. 000141, which cited the work of C.M. Richael, M. Kalyaeva, R.C. Chretien, H. Yan, S. Adimulam, A. Stivison, J.T. Weeks, and C.M. Rommens. 2008. Cytokinin vectors mediate marker-free and backbone-free plant transformation. Transgenic Res. 17: 905-917.
[5] The pSIM1678 plasmid was first described in BNF No. 000146.

followed by a copy of the *granule-bound starch synthase* promoter (both obtained from *S. tuberosum var. ranger russet*) in reverse orientation. These two convergent promoters drive the transcription of the *VInv* expression cassette. When transcribed, this entire sequence generates dsRNA containing inverted repeats consisting of partial sequences of *VInv* gene.

Following transformation, explants were grown in selection medium[6] and then grown in fresh medium that induced shoot development. Shoots that displayed cytokinin overproduction phenotype were discarded. Regenerated plantlets were grown for one to two months in a greenhouse prior to analysis of the transformed lines for insert number, insert integrity, and absence of vector backbone sequences.

Simplot used a combination of techniques, including Southern blot analysis, droplet digital polymerase chain reaction (ddPCR), and DNA sequencing to characterize the insertion event in Z6 potato. Genomic DNA samples from Z6 potato, V11 potato, and Snowden were digested with restriction enzymes prior to Southern blot analysis using a series of nine probes that span the length of the T-DNA region in pSIM1678. Simplot concludes that the data from this analysis demonstrate that Z6 potato contains a single intact copy of the T-DNA region of the pSIM1678 plasmid. Southern blot analysis using eight probes that span the full sequence of the backbone region of pSIM1678 plasmid were used to demonstrate that backbone DNA sequences were not present in Z6 potato. The number of T-DNA insertion sites and the number of inserts at each site was determined by ddPCR using undigested or *HphI* restriction digested genomic DNA. Simplot concludes that Z6 potato contains a single copy of the T-DNA region of pSIM1678 which was inserted at a single locus and confirmed that the previously inserted copy of the T-DNA region of pSIM1278 was present in Z6 potato. Simplot also concludes that backbone sequences from pSIM1678 were not present in Z6 potato.

Simplot used a combination of Southern blot analysis, whole genome sequencing (WGS), and Sanger DNA sequencing to characterize the junctions between inserted T-DNA region of pSIM1678 and flanking genomic sequences. Simplot estimated that average coverage of the T-DNA insert and flanking sequence for the WGS was 183x. Simplot stated that there was a 957 bp duplication of genomic DNA that flanked the inserted nucleotide sequence, which aligns to chromosome nine of the potato reference genome. Simplot stated that the genetic modification does not interrupt any known genes.

The stability of the inserted T-DNA sequences from pSIM1278 and pSIM1678 plasmids in Z6 potato was assessed by Southern blot analysis in plants that had undergone successive rounds of vegetative propagation.[7] Simplot conducted Southern blot analysis on genomic DNA obtained from leaves of Z6 plants (plantlets and three successive rounds of tuber propagation). Stability of the T-DNA insert from pSIM1278 plasmid was assessed using four probes and the T-DNA insert from pSIM1678 plasmid was assessed using four probes. Two of the probes hybridized to sequences that were present in both inserts. Simplot showed identical Southern blot hybridization patterns in Z6 plantlets and in plants that had undergone three successive rounds of tuber propagation.

[6] The selection media (M404, PhytoTechnology) contained timentin for inhibition of *A. tumefaciens*.
[7] Potatoes are propagated vegetatively and, thus, do not undergo meiotic recombination.

Simplot performed bioinformatics analyses using the nucleotide sequences of both T-DNA inserts 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. Simplot states that the results associated with the pSIM1278 insert in Z6 potato were the same as those obtained for V11 potato. Simplot identified putative peptides (>30 amino acids) that were associated with the pSIM1678 insert in Z6 potato. These putative peptides aligned with beta-fructofuranosidase precursor-vacuolar invertase peptide sequences in *S. lycopersicum*. Simplot concludes that these putative peptides do not raise safety concerns because they naturally occur in potatoes as part of the potato vacuolar invertase protein and the dsRNA sequence is unlikely to be translated into protein because the dsRNA is processed into short RNA interference transcripts. Based on the results of bioinformatics analyses, Simplot concludes that the T-DNA insertions do not lead to the production of putative polypeptides that would raise animal food safety concerns.

Intended Effects: Reduced Levels of Specific RNA Transcripts in Tubers

To characterize the levels of the five RNA transcripts targeted by the gene silencing constructs, Simplot performed Northern blot analyses on RNA isolated from tubers obtained from three replicates of field grown Z6 potato and Snowden (control) varieties. Simplot reported reductions in or absence of RNA transcripts for the *Asn1*, *Ppo5*, *PhL*, *R1*, and *VInv* genes in Z6 potato tubers when compared to control tubers. Based on these results, Simplot concludes that the decrease in RNA transcripts for the *Asn1*, *Ppo5*, *PhL*, and *R1* genes in Z6 potato is consistent with the results observed in V11 potato and that levels of *VInv* transcripts were decreased in Z6 potato tubers.

Animal Food Use

Simplot notes that in general, potatoes are used in human and animal food, for some industrial applications, and in propagation for subsequent plantings. Simplot states that raw potato waste from unprocessed potatoes, peels, and off-grade or discarded food items may be incorporated into animal food.

Composition

Scope of Analysis

Simplot conducted compositional analyses on tubers obtained from Z6 potato and the non-GE control variety, Snowden. The components selected for analysis were listed as key nutrients in the Organisation for Economic Cooperation and Development (OECD) potato consensus document.[8]

Study Design

Simplot grew Z6 potato and the control at four sites in the United States in 2018 using randomized complete block design with four replicates per site. The cultivation practices

[8] Organisation for Economic Cooperation and Development. 2002. Revised consensus document on compositional considerations for new varieties of potato (*Solanum tuberosum*): Key food and feed nutrients, toxicants, allergens, anti-nutrients, and other plant metabolites. Series on the safety of novel foods and feeds No. 4. ENV/JM/MONO(2002). OECD, Paris (this consensus document was withdrawn in 2020 and replaced by revised consensus document No. 33).

were location specific, based on recommendations from regional specialists. Simplot randomly selected six medium-sized tubers with skin from each replicate. Tuber samples were ground with liquid nitrogen into a powder using an industrial blender and stored at -70°C until compositional analyses were conducted.

Simplot determined the levels of proximates (moisture, crude protein, total fat, ash, crude fiber, and carbohydrates (by calculation)), calories (by calculation), vitamins (B_3, B_6, and C), minerals (copper, magnesium, and potassium), 18 amino acids, and glycoalkaloids (alpha-solanine and alpha-chaconine). To assess the efficacy of intended effects, Simplot also determined the levels of sucrose and reducing sugars (fructose plus glucose) in tubers.

Simplot statistically compared each component for Z6 potato with the control across locations using a mixed model analysis of variance with location and replicate within location as random variables. Simplot statistically compared values of measured components in Z6 potato to the control with a threshold for statistical significance of $P \leq 0.05$. When statistical differences were observed between Z6 potato and control, Simplot determined whether the mean value for the component in Z6 potato fell within the range of values in the scientific literature; hereafter referred to as combined reference range. For proximates, minerals, vitamins and amino acids, Simplot referred to data presented in International Life Sciences Institute 2019 Crop Composition Database (ILSI CCD) and OECD consensus document. For glycoalkaloids, Simplot referred to Kozukue and coworkers (2008)[9], and for sucrose and fructose plus glucose, Simplot referred to Amrein and coworkers (2003) and Vivanti and coworkers (2006)[10]

Results of Analyses

Simplot reported statistically significant differences in the levels of carbohydrates (by calculation), calories (by calculation), and moisture between Z6 potato and control. Simplot also reported that vitamins B_3 and C were statistically higher in Z6 potato when compared to control. Although total crude protein was not significantly different between Z6 potato and control, the concentrations of most of the amino acids were statistically higher (histidine and tryptophan were not different), whereas aspartic acid +asparagine was statistically lower in Z6 potato when compared to the control. Simplot found that the mean values for all of these components in Z6 potato and the control fell within the combined reference range. Simplot reports that total glycoalkaloid (sum of alpha-solanine and alpha-chaconine) concentrations were statistically lower in Z6 potato when compared to control (11.8 versus 13.9 milligrams/100 grams wet weight).[11] Simplot concludes that Z6 potato is nutritionally equivalent to conventional potatoes.

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[9] Kozukue, N., K.-S. Yoon, G.-I. Byun, S. Misoo, C.E. Levin, and M. Friedman. 2008. Distribution of glycoalkaloids in potato tubers of 59 accessions of two wild and five cultivated *Solanum* species. J. Agric. Food Chem. 56: 11920-11928.

[10] Amrein, T.M., S. Bachmann, A. Noti, M. Biedermann, M.F. Barbosa, S. Biedermann-Brem, K. Grob, A. Keiser, P. Realini, F. Escher, R. Amado. 2003. Potential of acrylamide formation, sugars, and free asparagine in potatoes: A comparison of cultivars and farming systems. J. Agric. Food Chem. 51:5556-5560; Vivanti, V. E. Finotti, and M. Friedman. 2006. Level of acrylamide precursors asparagine, fructose, glucose, and sucrose in potatoes sold at retail in Italy and in the United States. J. Food Sci. 71:C81-C85.

[11] Simplot indicates the safe limit for glycoalkaloids in tubers is 20 milligrams/100 grams fresh weight.

Intended Compositional Change – Reduction in Reducing Sugars

Simplot evaluated Z6 potato for the expected outcome of silencing the *PhL*, *R1*, and *VInv* genes, i.e., decreased levels of reducing sugars. Simplot measured concentration of sucrose and reducing sugars (fructose and glucose) in potatoes at harvest and after storage at 7°C for six months. Simplot reported that the concentration of reducing sugars were statistically lower in stored Z6 potato and tended to be lower in fresh Z6 potato when compared to control. The concentration of sucrose was statistically higher in fresh Z6 potato when compared to control and there was no significant difference in sucrose levels in potatoes stored for six months. Simplot notes that alteration of the type of sugars (reducing sugars versus sucrose) is a measure of tuber quality rather than safety.[12] In addition, Simplot demonstrated that total aspartic acid + asparagine concentrations were lower in Z6 potato when compared to the control, an intended effect of the *Asn1* gene silencing.

Summary of Compositional Analyses

Simplot states that Z6 potato is compositionally equivalent to Snowden, a commercially grown potato variety, and Z6 potato is as safe and nutritious for use in animal food as conventional potato varieties that have a long history of safe use in animal food.

Conclusion

FDA evaluated Simplot's submission to determine whether Z6 potato raises any safety or regulatory issues with respect to the intended modifications or with respect to its use in animal food. Based on the information provided by Simplot 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.

Simplot concludes that animal food derived from Z6 potato is as safe as and is not materially different in composition, safety, or any relevant parameter from other potato varieties now grown, marketed, and consumed. At this time, based on Simplot's data and information, the agency considers Simplot's consultation on Z6 potato to be complete.

Rial A. Christensen -S

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Date: 2021.08.13 09:24:46 -04'00'

Rial Christensen, Ph.D.
Animal Scientist

[12] Sucrose is typically converted in the digestive tract of animals to fructose and glucose.