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CFSAN Note to the File

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To: Administrative Record, BNF No. 000178

Subject: *Del/Ros1*-N tomato

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Summary

Norfolk Plant Sciences (NPS) has completed a consultation with the Food and Drug Administration (FDA) on food derived from *Del/Ros1*-N tomato genetically engineered to express Delila and Rosea1 transcription factors to induce anthocyanin biosynthesis in the fruit, and the NPTII protein as a selectable marker. This document summarizes NPS'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 of this tomato.

Based on the safety and nutritional assessment NPS has conducted, it is our understanding that NPS 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 *Del/Ros1*-N tomato is comparable to and as safe as human food from other tomato varieties.

CFSAN evaluated data and information supporting these conclusions and considered whether *Del/Ros1*-N tomato 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 *Del/Ros1*-N tomato.

Subject of the Consultation

Crop	Tomato
Designation	*Del/Ros1*-N

Intended trait	Increased levels of anthocyanins in the fruit
Developer	Norfolk Plant Sciences
Submission received	March 3, 2020
Amendment(s) received	May 27, 2021; June 24, 2022; November 10, 2022; April 7, 2023; May 25, 2023
Intended use	For general use in human food
Transformation plasmid	pDEL.ROS
Expression cassettes 1&2	*Del* and *Ros1* genes encoding Delila and Rosea1 transcription factors from *Antirrhinum majus* each under the control of a fruit specific E8 promoter from tomato to induce anthocyanin biosynthesis in tomato
Expression cassette 3	*nptII* gene encoding neomycin phosphotransferase II (NPTII) derived from *Escherichia coli* transposon Tn5 to confer antibiotic resistance for use as a selectable marker
Method for conferring genetic change	*Agrobacterium*–mediated transformation

Molecular Characterization

Breeding and selection

NPS transformed tomato (*Solanum lycopersicum cv*. MicroTom) plants and selected the primary transformant with strongest anthocyanin accumulation (referred to as *Del/Ros1*-N) for developing the homozygous *Del/Ros1*-N trait in MicroTom and MoneyMaker genetic backgrounds through breeding. According to NPS, *Del/Ros1*-N in MoneyMaker was produced by crossing a plant from the T1 generation of *Del/Ros1*-N with MoneyMaker to generate a purple fruited F1 population. One of the F1 plants was selfed and a dark purple F2 plant was selfed through eight generations propagated by single seed descent to develop the stock line *Del/Ros1*-N in MoneyMaker. To develop *Del/Ros1*-N in MicroTom, the same T1 plant of *Del/Ros1*-N plant used to make *Del/Ros1*-N in MoneyMaker was selfed across six generations using single seed descent to generate the stock line *Del/Ros1*-N in MicroTom. Plants from *Del/Ros1*-N in MoneyMaker and MicroTom stock lines were used for characterization of the T-DNA insertion, segregation analysis, and compositional analysis.

Confirmation of intended genetic change

NPS used quantitative PCR and Southern blot analysis to confirm the presence of a single DNA insertion in both *Del/Ros1*-N in MicroTom and *Del/Ros1*-N in MoneyMaker stock lines. Using high throughput sequencing, NPS amplified the *Del* and *Ros1* genes in a *Del/Ros1*-N MicroTom tomato and confirmed the integrity of the insertion. NPS used inverse PCR to assess the T-DNA flanking sequences and found that there is a 94 bp deletion in the tomato genome at the insertion site, a 52 bp deletion at the T-DNA right border (RB) region and a 75 bp deletion at the T-DNA left border (LB) region.

Absence of vector backbone DNA

NPS analyzed high throughput sequencing data from a *Del/Ros1*-N tomato for the presence of sequences from the vector backbone. NPS did not detect any vector backbone sequences.

Inheritance and stability

NPS used PCR and sequence comparison of DNA samples from T1 versus T6 MicroTom plants and F9 MoneyMaker versus T6 MicroTom plants to confirm the stability of the insertion across generations and genetic backgrounds. NPS stated that the purple phenotype was inherited in a Mendelian segregation fashion in all tomato genetic backgrounds. The purple tomato trait has been introgressed into other tomato varieties including Ailsa Craig, Lucinda, VF36, and Ohio 8423 (an industrial processing tomato variety for tomato juice production), which confirmed the stability of the phenotype.

Open reading frame analysis

NPS used bioinformatic analyses to assess whether any open reading frames (ORFs) were generated or disrupted as a result of the DNA insertion and if so, whether the putative protein translation of any new ORFs raised toxicity or allergenicity concerns relevant to human food. NPS searched the genomic DNA sequences flanking the insertion in *Del/Ros1*-N tomatoes and found no known tomato ORFs were interrupted by the presence of the *Del/Ros1*-N insertion. NPS also assessed the junction sequences for putative new peptides of equal or greater than 30 amino acids created at the insertion site of the T-DNA in *Del/Ros1*-N tomatoes. One putative peptide was identified and analyzed; however, this peptide has no homology to any known allergen or protein and there was no evidence this sequence is transcribed in tomato. NPS concludes the results from the open frame analysis do not raise food safety concerns.

Introduced Proteins: Delila and Rosea1 Transcription Factors

Intended trait	Increased levels of anthocyanins in the fruits
Source organism	*Antirrhinum majus* (Snapdragon)
Protein description	Delila is a basic helix-loop-helix transcription factor and Rosea1 is a R2R3MYB-related transcription factor.
Intended function	Delila and Rosea1 activate a broad spectrum of genes encoding enzymes required for anthocyanins biosynthesis, side-chain modification, and likely transportation of anthocyanins into the vacuole.

NPS used Orbitrap Liquid Chromatography-Mass Spectrometry (LC-MS) to measure the concentration of Delila and Rosea1 proteins in the juice made from *Del/Ros1*-N tomatoes grown in greenhouses. NPS stated neither Delila nor Rosea1 proteins were detected in the juice from *Del/Ros1*-N tomatoes.[1] Considering the similar processes to prepare samples for measuring proteins in tomato fruit and tomato juice, NPS stated Delila and Rosea1 proteins would similarly be below detection levels in whole fruit. NPS noted that the limit of detection of mass

[1] NPS notes the juice production was done according to the commercial standards with a “cold break” process that does not risk heat-degradation of proteins.

spectrometry is calculated as less than 0.2 ng Rosea1 protein and less than 0.5 ng of Delila protein per milliliter juice.

NPS used bioinformatic analysis to assess Delila and Rosea1 for sequence similarity to known allergens and toxins. NPS found no sequence similarity to known allergens using the ADFS database and AllergenOnline database,[2] and no sequence similarity to known toxins using the T3DB database.[3] NPS explained that the transcription factors Delila and Rosea1 in *Del/Ros1*-N tomatoes are functionally and structurally equivalent to transcription factors that control anthocyanin biosynthesis in commonly consumed fruits and vegetables with a history of safe use.

NPS assessed the stability of Delila and Rosea1 proteins to digestive enzyme using a validated method. NPS found Delila and Rosea1 proteins are digested by pepsin in simulated gastric fluids within 30 seconds and 10 minutes respectively.

Introduced Protein: Neomycin phosphotransferase II (NPTII)

Intended trait	Resistance to aminoglycoside antibiotics, for use as a selectable marker during genetic engineering
Source organism	*Escherichia coli* transposon Tn5
Protein description	Aminoglycoside phosphotransferase
Intended function	Phosphorylation of aminoglycoside antibiotics

NPS used Orbitrap LC-MS to measure the concentration of NPTII protein in the juice made from *Del/Ros1*-N tomatoes. NPS stated NPTII protein was not detected in the juice. NPS noted that the limit of detection of LC-MS analysis is calculated as less than 0.2 ng NPTII protein per milliliter juice. Moreover, NPTII is authorized for use as a food additive in tomato (see 21 CFR 173.170; NPTII is also referred to as aminoglycoside 3′ phosphotransferase II in FDA's regulations).

Human Food Nutritional Assessment

Intended human food uses

Tomato is consumed fresh, in salads, as well as a processed food. Examples of processed products are tomato juice, paste, puree, ketchup and sauce. The intended uses of *Del/Ros1*-N tomatoes in human food are the same as other tomatoes on the US market.

Intended trait

Del/Ros1-N tomatoes were developed to produce increased levels of anthocyanins. The two predominant anthocyanin species in *Del/Ros1*-N tomatoes are delphinidin 3-*O*-(coumaroyl)

[2] NPS defined significant homology to allergens as >35% identity over 80 amino acids, ≥ 8 contiguous, identical amino acids, or overall homology to a known allergen. These databases are accessible at http://allergen.nihs.go.jp/ADFS/index.jsp?pagen=about and http://allergenonline.org/.
[3] http://www.t3db.ca/

rutinoside-5-*O*-glucoside and petunidin 3-*O*-(coumaroyl) rutinoside-5-*O*-glucoside with some other anthocyanin species at lower levels in the ripened tomato fruit.

Safety assessment of anthocyanins in human food

NPS states that anthocyanins accumulated in *Del/Ros1*-N tomatoes are the same as those that accumulate in the skin of some purple-skinned tomato varieties that are already available on the market, and in eggplant and purple-fleshed potatoes.

NPS compared the levels of the anthocyanins in *Del/Ros1*-N tomatoes and non-transformed parental red tomatoes (control). NPS states that anthocyanins are undetectable in the control but averaged 2.83±0.46 mg anthocyanins per g fresh weight in hemizygous *Del/Ros1*-N MicroTom tomatoes. NPS reports that the levels of anthocyanins in *Del/Ros1*-N MoneyMaker tomatoes were 0.4 mg anthocyanin per g fresh weight.[4]

NPS assessed the dietary exposure to anthocyanins from consumption of *Del/Ros1*-N tomatoes assuming complete replacement of red tomatoes in the human diet with the *Del/Ros1*-N tomatoes. NPS used the 2009-2010 cycle of National Health and Nutrition Examination Survey (NHANES) data to estimate 2-day consumption of all foods containing tomatoes with recipe-specific levels of tomatoes in each food. NPS estimated a dietary exposure to anthocyanins in *Del/Ros1*-N tomatoes to be 100 mg/day at the mean, and 225 mg/day at the 90th percentile. NPS states these estimates of dietary exposure to anthocyanins in *Del/Ros1*-N tomatoes are of the same order of magnitude as that of consumption of high anthocyanin foods.[5] Since the level of anthocyanin in *Del/Ros1*-N tomatoes (0.3%) is similar to that in high anthocyanin foods (e.g., 0.5% in blueberry), the consumption of foods containing *Del/Ros1*-N tomatoes is comparable to the consumption of high anthocyanin foods. For instance, consumption of a serving of *Del/Ros1*-N tomato juice (8 ounces or 243 g) is equivalent to the consumption of about 2 servings of blueberries (1 cup or 148 g).

Analysis of key nutrients, anti-nutrients, and toxicants

To ensure the absence of unintended changes to the levels of components relevant to safety or nutrition, NPS analyzed *Del/Ros1*-N tomatoes (in MoneyMaker genetic background) and the control, grown in a greenhouse. Five *Del/Ros1*-N tomato plants and five control plants were grown in alternating rows. Fruit collected from a single plant was pooled and treated as one replicate. The compositional analyses were performed based on the principles outlined in the Organisation for Economic Co-operation and Development (OECD) Consensus Document for tomato composition.[6] NPS analyzed and reported the levels of components in the *Del/Ros1*-N tomato and the control, including proximates (protein, fat, carbohydrates, fiber, and ash), fatty

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[4] In the amendment of November 10, 2022, NPS provided another measurement at 0.17 mg per g fresh fruit in the MoneyMaker genetic background.

[5] FDA estimated dietary exposure to anthocyanins from consumption of *Del/Ros1*-N tomatoes using 2011-2018 NHANES to be comparable to estimates of dietary exposure to anthocyanins from consumption of high anthocyanin foods, also using 2011-2018 NHANES survey.

[6] OECD, 2008. Consensus document on compositional considerations for new varieties of tomato: key food and feed nutrients, toxicants and allergens. ENV/JM/MONO(2008)26. Organisation for Economic Co-operation and Development, Paris, France.

acids, minerals (magnesium, potassium, and sodium), carotenoids (β-carotene and lycopene), vitamins (vitamin C, vitamin K1, and folate), and α-tomatine.

NPS notes that the levels of most nutritional components in the *Del/Ros1*-N tomato and the control are similar or with minor differences; the levels are 25% higher for total folate, lycopene, β-carotene, and α-tomatine in the *Del/Ros1*-N tomato compared to the control. NPS notes, however, that the levels of all tested components in the *Del/Ros1*-N tomato and the control are within the ranges reported in the literature.[7]

NPS also analyzed and compared the levels of components in tomato juice from the *Del/Ros1*-N tomato (in Ohio 8423 genetic background) and non-transformed wildtype Ohio 8423 control tomato and concludes that the juice from the *Del/Ros1*-N tomato is similar to that of the control, and the nutritional composition of the juice is similar to that of whole tomato fruit. NPS concludes that *Del/Ros1*-N tomato is as safe and as nutritious as other tomatoes on the market.

Human Food Labeling Considerations

It is a producer's or distributor's responsibility to ensure that labeling of the foods it markets derived from *Del/Ros1*-N tomato 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 *Del/Ros1*-N tomato may be used in various food applications. Depending on the particular food application, the increased levels of anthocyanins and therefore, purple colored-fruit may be considered material facts requiring disclosure under Sections 201(n) and 403(a)(1) of the FD&C Act [21 U.S.C. § 321(n) and 343(a)(1)]. Companies marketing food from *Del/Ros1*-N tomato 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 *Del/Ros1*-N tomato and products produced from it. Failure to do so may result in misbranding of products produced from *Del/Ros1*-N tomato within the meaning of Sections 201(n) and 403(a)(1) of the FD&C Act.

Conclusion

Based on the information provided by NPS 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 *Del/Ros1*-N tomato. We consider the consultation with NPS on *Del/Ros1*-N tomato to be complete.

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[7] NPS states that the higher concentrations of α-tomatine in the *Del/Ros1*-N tomato compared to the control are consistent with the slower ripening of the *Del/Ros1*-N tomato.