Simplot PLANT SCIENCES

Simplot Plant Sciences
5369 W. Irving Street
Boise, Idaho 83706

June 16, 2020

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Road, Unit 98
Riverdale, MD 20737

Re: Confirmation that low PPO avocados developed with CRISPR/Cas9 are not regulated articles

Dear Ms. Juarez,

J.R. Simplot Company's Plant Sciences division (Simplot) respectfully seeks confirmation from Biotechnology Regulatory Services that low PPO avocados (*Persea americana* Mill.) developed with CRISPR/Cas9 do not meet the definition of a regulated article under 7 CFR Part 340.

USDA has previously reviewed the low PPO trait in a tree crop, apple (*Malus* × *domestica*), and determined that non-browning apples modified to reduce polyphenol oxidase are unlikely to pose a plant pest risk[1,2].

Simplot has developed a Ribonucleoprotein (RNP) method that enables delivery of CRISPR/Cas9 elements into plant cells. The method results in double-stranded breaks within the alleles of a targeted gene and knocks out both alleles in **[ ]** *Ppo* **[ ]**. The final selected lines do not contain any introduced DNA from the CRISPR/Cas9. With this method, gene editing is accomplished by transfection of avocado protoplast cells with ribonucleoprotein (RNP) complexes consisting of purified CAS9 protein bound to a synthetic guide RNA (gRNA) (Andersson et al., 2018). **CBI Deleted**

Avocados are not a plant pest and do not pose a weed potential. Low PPO avocados have no introduced plant pest sequences, and none are used in the RNP method. It is unlikely that low PPO avocados developed with CRISPR/Cas9 will become a plant pest or have altered weed potential compared to conventional avocados. Therefore, low PPO avocados do not meet the definition of a regulated article based on 7 CFR Part 340.

Intended Phenotype

Low PPO avocados have reduced polyphenol oxidase (PPO) protein, an enzyme that is responsible for enzymatic darkening and discoloration in avocado fruit flesh (mesocarp tissue). When avocados are mashed or cut, PPO enzymes are released by the disruption of the cell structure, resulting in unappealing discoloration. The discoloration of flesh is undesirable for both processors and consumers, resulting in decreased fruit quality and food waste.

[1] https://www.aphis.usda.gov/brs/aphisdocs/10_16101p_fea.pdf
[2] https://www.aphis.usda.gov/brs/aphisdocs/16_00401p_det_pprsa.pdf

Intended Activity

It is Simplot's intention to make low PPO avocados developed with CRISPR/Cas9 and to introduce them into the market.

Development of Low PPO Avocados

Simplot is using CRISPR/Cas9 to generate one or more targeted DNA double-stranded breaks within the coding region of **[ ]** *Ppo* **[ ]**. No external repair template is provided. Instead, the plant's own cellular mechanism repairs the breaks, resulting in small nucleotide deletions, additions, or substitutions at the break sites on each allele. Both alleles of each *Ppo* gene will likely be repaired by the plant in a slightly different way. **CBI Deleted**

Given that commercial avocados are diploids and prone to discoloration, mutations in a pair of homologous alleles of **[ ]** *Ppo* **[ ]** would result in a desirable reduction of PPO activity. Plants are molecularly screened for double-allele knockouts in each of **[ ]** *Ppo* **[ ]**, which allows for selection of plants with the desired reduction of PPO activity in fruit flesh. **CBI Deleted** **CBI Deleted** **CBI Deleted**

RNP Method

This CRISPR/Cas9 method is based on transfection of avocado protoplasts with ribonucleoprotein (RNP) complexes, which consist of purified CAS9 protein bound to a synthetic gRNA (Andersson et al., 2018). This method does not rely on the introduction of DNA to serve as a transcriptional template, nor is an external repair template provided. Protoplasts are transfected to deliver CRISPR/Cas9 components into plant cells, generating double-stranded breaks within **[ ]** *Ppo* **[ ]**. The cell's natural DNA repair mechanism then repairs the break by non-homologous end-joining, which can lead to knockout of the targeted protein function (Figure 1). **CBI Deleted**

Protoplasts are regenerated through a callus phase into plants using regeneration medium. Plants are then grown in tissue culture where they are screened molecularly for the desired genotype before being moved to the greenhouse or field to select for plants with the desired phenotype. Plants containing the desired targeted knockout and phenotype will be selected for further development.

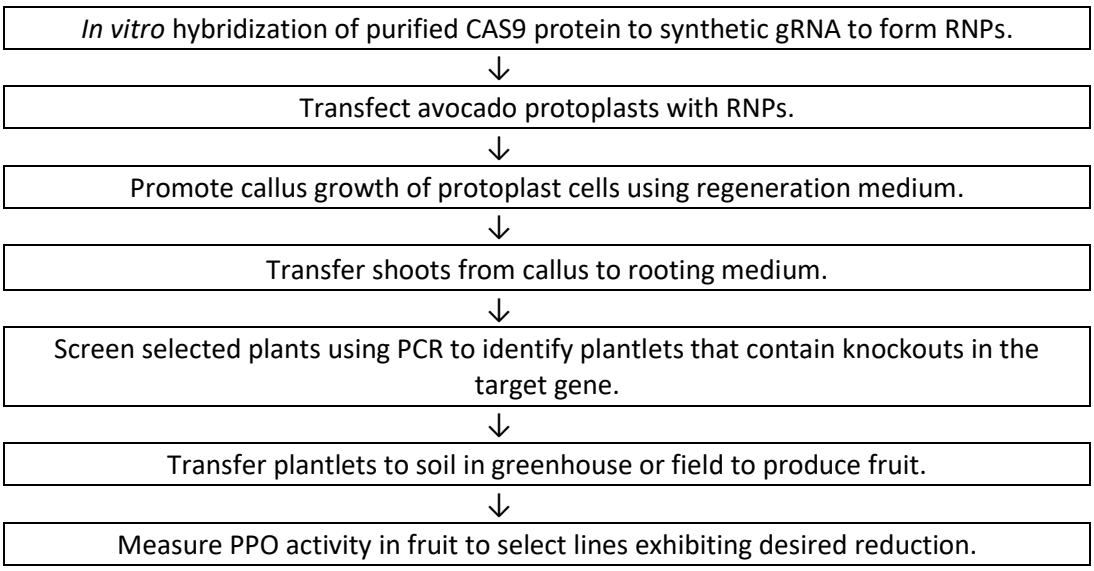

Figure 1. Schematic of CRISPR/Cas9 RNP Method

Avocado is Not a Regulated Article

Avocado (*Persea americana* Mill.) is not a federal noxious weed pursuant to 7 CFR 360 or reported as a pest or weed in managed ecosystems and is not recorded as being invasive of natural ecosystems. Avocado is a species that was introduced into the U.S. for fruit cultivation and native to Central America. Gene flow from cultivated avocado to wild relatives is considered unlikely as there are no known sexually compatible relatives of avocado in the United States (Williams, 1976).

Previous Relevant Am I Regulated Letters

There are two previous Am I Regulated letters describing multiple-allele knockouts of *Ppo* in potatoes using TALENS, a gene editing tool that is similar to CRISPR/Cas9 (Table 1). In both cases, USDA concluded that the low PPO potatoes would not be regulated articles because they contained no introduced material, potato is not a Federal noxious weed, and the trait would not increase weediness. This would be the same outcome for avocado in that there will be no introduced material, avocado is not a Federal noxious weed, and the PPO knockout trait would be unlikely to increase weediness.

Table 1. Previous AIR letters for PPO Knockouts in Potatoes

Company	Topic	Date of USDA Response	Outcome
Simplot	PPO5 knockout in multiple alleles of potato using TALENS	Dec. 2, 2016	Not regulated
Calyxt	PPO knockout in multiple alleles of potato using TALENS	Sept. 15, 2016	Not regulated

Deregulated Events with Down Regulated PPO

USDA-APHIS has previously assessed the plant pest potential from down-regulation of PPO in 14 potato events and 3 apple events, resulting in the deregulation of 17 events. These events possess lower PPO activity from RNAi:

- E12, F10, J3, and others (10 events total), 13-022-01p[3];
- W8, 14-093-01p[4];
- V11, 15-140-01p[5];
- X17 and Y9, 16-064-01p[6];
- GS784 and GD743 (4 *Ppo* genes were silenced), 10-161-10p[7]; and
- NF872 (4 *Ppo* genes were silenced), 16-004-01p[8].

In the plant pest risk assessments for these petitions, USDA stated that the events were not observed to consistently exhibit any increase in susceptibility to plant pathogens or pests as a result of the reduction in PPO activity. USDA concluded:

- The events pose no more of a plant pest risk than their respective parental varieties or other conventional varieties;
- The genetic modification in the events is not expected to increase the potential for gene flow, hybridization and/or introgression to occur to sexually compatible relatives compared to the nontransgenic recipient or other varieties of the crop commonly grown; and
- The deregulated events are unlikely to persist as a troublesome weed or to have an impact on current weed management practices.

[3] https://www.aphis.usda.gov/brs/aphisdocs/13_02201p_ppra.pdf
[4] https://www.aphis.usda.gov/brs/aphisdocs/14_09301p_fpra.pdf
[5] https://www.aphis.usda.gov/brs/aphisdocs/15_14001p_det.pdf
[6] https://www.aphis.usda.gov/brs/aphisdocs/16_06401p_det_pprsa.pdf
[7] https://www.aphis.usda.gov/brs/aphisdocs/10_16101p_fpra.pdf
[8] https://www.aphis.usda.gov/brs/aphisdocs/16_00401p_det_pprsa.pdf

No Plant Pest Sequences in Low PPO Avocados

With the RNP method, nothing remains in the final selected edited plants but the naturally repaired double-stranded breaks, which result in the knockout of PPO.

Conclusions

Simplot has developed an RNP CRISPR/Cas9 method to create double-stranded breaks in **[ ]** avocado *Ppo* **[ ]**. The final selected lines do not contain any introduced DNA from the CRISPR/Cas9 system. Low PPO avocados developed with CRISPR/Cas9 contain no plant pest sequences, and the phenotype is highly unlikely to result in increased weediness or plant pest potential.

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Simplot requests confirmation from Biotechnology Regulatory Services that Simplot's low PPO avocados (*Persea americana* Mill.) developed with CRISPR/Cas9 do not meet the definition of a regulated article under 7 CFR Part 340.

Sincerely,

Craig Richael, Ph.D.
Director of Research and Development

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References

Andersson, M., Turesson, H., Olsson, N., Fält, A.S., Ohlsson, P., Gonzalez, M.N., Samuelsson, M., Hofvander, P., 2018. Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. Physiol. Plant. 164, 378–384. https://doi.org/10.1111/ppl.12731

Williams, L.O., 1976. The botany of the avocado and its relatives. Proc. Int. Trop. Fruit Short Course. Avocado. Miami Beach, Florida 9–15.