Simplot
PLANT SCIENCES

November 11, 2016

Dr. Michael J. Firko
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Road, Unit 98
Riverdale, MD 20737

Re: Confirmation that low PPO5 potatoes are not regulated articles

Dear Dr. Firko,

Simplot Plant Sciences (Simplot) respectfully seeks confirmation from Biotechnology Regulatory Services that low PPO5 potatoes (*Solanum tuberosum,* L.) do not meet the definition of a regulated article under 7 CFR Part 340. Simplot has developed a method using disarmed *Agrobacterium* to transiently deliver transcriptional activator-like effector nuclease (acronym: TALEN) into plant cells, generating targeted knockouts in the native potato *Ppo5* gene. Low PPO5 potatoes developed using this method will produce reduced PPO5 protein, a polyphenol oxidase (PPO) enzyme that is responsible for black spot, enzymatic darkening and discoloration in potato tubers. The end product will be like a null segregant in that no genetic material used to create the knockouts is present in the final product.

Potatoes are not a plant pest and do not pose a weed potential. The method used to generate the targeted *Ppo5* knockouts results in plants with no introduced plant pest sequences, and the knockouts will not generate a plant pest or pose a weed potential. Similar to Simplot's previously deregulated events, there is no basis to conclude that low PPO5 potatoes will become a plant pest or have altered weed potential compared to conventional potatoes. Therefore, low PPO5 potatoes do not meet the definition of a regulated article based on 7 CFR Part 340.

**Intended Phenotype**

The intended phenotype of low PPO5 potatoes is reduced black spot. When potatoes are bruised or damaged, PPO enzymes can be released from the plastids resulting in discolored tubers or 'black spot'. Black spot is a post-harvest physiological phenomenon resulting from the handling of potato tubers during harvest, transport, storage, and processing. The discoloration of tubers is undesirable for both processors and consumers.

Reducing PPO levels in potatoes can decrease the occurrence of black spot, resulting in increased tuber quality and less food waste (Halterman et al., 2016). An estimated 1.9 million metric tons of bruise losses occurred at the grower, packer, retailer and foodservice levels of the market chain in the United

States in 2013 (Halterman et al., 2016). A 2008 study estimates that 35% of fresh potatoes were lost as food waste at the retail and consumer levels of the market chain in the United States (Buzby et al., 2011); a significant portion of these losses would be attributable to black spot bruising.

A family of genes encoding PPO exists in potato, with *Ppo5* (also known as *pot32*) being the most highly expressed in tubers (Thygesen et al., 1995). Because potato is a tetraploid, it is likely that events with knockouts in 3-4 of the four *Ppo5* alleles will be needed for sufficient reduction of PPO in tubers.

**Intended Activity**

Upon confirmation from APHIS-BRS that low PPO5 potatoes are not regulated, Simplot, after first confirming that no introduced DNA remains, intends to conduct interstate movement and field releases of these potatoes.

**Development of Low PPO5 Potatoes**

The method described here incorporates TALENs into a binary transformation vector to generate targeted knockouts in the potato *Ppo5* gene. TALEN is a site-directed nuclease comprised of the FokI restriction endonuclease fused to the DNA binding domain of *Xanthomonas*-derived TAL-effector. TALEN technology is a method of targeted base pair alteration that is functionally equivalent to other methods such as meganucleases and zinc-finger nucleases (Gaj et al., 2013).

The binary vector, pSIM 3337, encodes four expression cassettes (Table 1):

- Two TALEN cassettes, one with a potato polyubiquitin promoter (pUBI7) and one with a figwort mosaic virus (FMV) promoter;
- A kanamycin selectable marker cassette ($Kan^R$); and
- An isopentenyl transferase (ipt) cassette.

The binary vector contains the vector backbone for replication in bacteria and the *Agrobacterium* T-DNA right border sequence.

Targeted knockouts using TALENs are made using *Agrobacterium* transformation to introduce the binary vector into potato cells. After delivery of the vector, kanamycin selection will be used to eliminate untransformed cells that did not receive the $Kan^R$ expression cassette (Screen 1; Figure 1). Explants will be moved to kanamycin-free medium to regenerate cells containing the introduced DNA.

The vector lacks a T-DNA left border, allowing stable or transient transfer of the entire binary vector into potato cells. Regenerated plants that contain a stable integration of the ipt cassette overexpress the *ipt* gene, leading to abnormal plant growth. These plants will be eliminated from further development (Screen 2, Figure 1). Plants generated from the transient expression of *ipt* have a normal plant growth phenotype and will be advanced for further analysis (Richael et al., 2008).

Similar to the transient expression of the $Kan^R$ and ipt cassettes, transient expression of the TALEN cassettes will result in targeted *Ppo5* knockouts and the desirable phenotype. TALEN DNA recognition sequences are coupled with the site-specific nuclease, FokI, to create double-stranded breaks in the

*Ppo5* potato gene. The cells' natural DNA repair mechanism then repairs the break by non-homologous end-joining. DNA repair by non-homologous end-joining occasionally produces small deletions, insertion of a few nucleotides, or a single nucleotide mutation in the targeted gene that lead to knockout of the targeted protein function.

To confirm absence of the binary vector and presence of the desired gene knockout, selected plants will be screened using PCR, T7 endonuclease I assay, and Southern blot analysis. Only plants lacking all elements of the binary vector and containing the desired targeted knockout in *Ppo5* will be selected for commercial development.

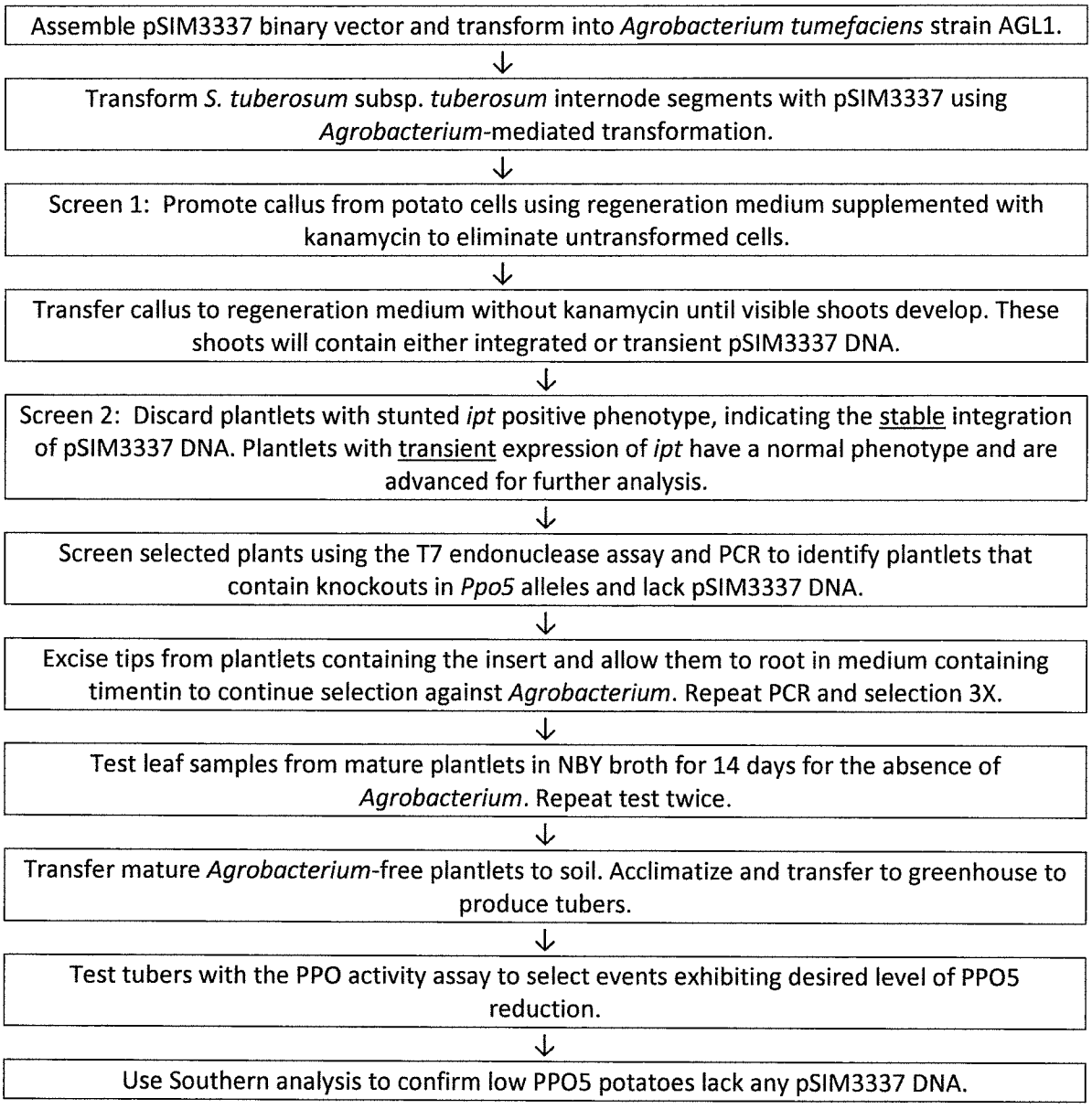


**Figure 1. Schematic of Transient Transformation with pSIM3337**

**Table 1. Genetic Elements in pSIM3337**

| | Genetic Element | Source | Function |
|---|---|---|---|
| Cassette 1 | Polyubiquitin promoter (pUbi7) | *Solanum tuberosum* | Drives expression of the TAL effector and Fok1 endonuclease in potato cells |
| | TAL Effector 1 | *Xanthomonas* spp. | An array of 34 amino acid DNA-binding motifs that specifically recognizes target sequence within the *Ppo5* gene |
| | Fok1 | *Flavobacterium okeanokoites* | Gene for a bacterial type IIS restriction endonuclease consisting of an N-terminal DNA-binding domain and a non-specific DNA cleavage domain that cleaves potato genomic DNA downstream of the binding site |
| | nos Terminator | *Agrobacterium tumefaciens* | Nopaline synthetase terminator for TAL effector and Fok1 endonuclease gene transcription |
| Cassette 2 | FMV Promoter | Figwort Mosaic Virus | Drives expression of the TAL effector and Fok1 endonuclease in potato cells |
| | TAL Effector 2 | *Xanthomonas* spp. | An array of 34 amino acid DNA-binding motifs that specifically recognizes target sequence within the *Ppo5* gene |
| | Fok1 | *Flavobacterium okeanokoites* | Gene for a bacterial type IIS restriction endonuclease consisting of an N-terminal DNA-binding domain and a non-specific DNA cleavage domain that cleaves potato genomic DNA downstream of the binding site |
| | ocs Terminator | *Agrobacterium tumefaciens* | Octopine synthetase gene terminator for TAL effector and Fok1 endonuclease gene transcription |
| Cassette 3 | Polyubiquitin promoter (pUbi7) | *Solanum tuberosum* | Drives expression of $Kan^R$ cassette in potato cells |
| | Aminoglycoside phosphotransferase gene | *E. coli* | Kanamycin resistance gene used as a selectable marker in plant transformation |
| | Terminator of the ubiquitin-3 gene (tUbi3) | *Solanum tuberosum* | Terminator for $Kan^R$ gene transcription |
| Cassette 4 | Polyubiquitin promoter (pUbi3) | *Solanum tuberosum* | Drives expression of the *ipt* gene in potato cells |
| | Isopentenyl transferase (*ipt*) gene | *Agrobacterium tumefaciens* Ti-plasmid | IPT catalyzes the condensation of AMP and isopentenyl-pyrophosphate to form isopentenyl-AMP, a cytokinin in the plant. Overexpression results in abnormal growth phenotypes in plant. |
| | Terminator of the ubiquitin-3 gene (tUbi3) | *Solanum tuberosum* | Terminator for *ipt* gene transcription |
| | Right Border | synthetic | Primary cleavage site |
| | Overdrive | *Agrobacterium tumefaciens* Ti-plasmid | Enhances cleavage at the Right Border site |
| | pVS1 partitioning protein StaA (PVS1 Sta) | *Pseudomonas aeruginosa* pVS1 | pCAMBIA1301 sequence resulting in pVS1 stability |
| | pVS1 replicon (pVS1Rep) | *Pseudomonas aeruginosa* pVS1 | pCAMBIA1301 pVS1 replication region in *Agrobacterium* |
| | pBR322 bom | pBR322 | pCAMBIA1301 - mobilization of plasmid DNA from *E. coli* to *A. tumefaciens* |
| | pBR322 Origin of replication (pBR322 ori) | pBR322 | pCAMBIA1301 - bacterial origin of replication |
| | Aminoglycoside-3'-adenyltransferase (aadA) | *Escherichia coli* | Spectinomycin and streptomycin resistance gene used as a selectable marker for *E.coli* and *Agrobacterium* transformation |

**Potato is Not a Regulated Article**

Potato (*Solanum tuberosum,* L.), 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. Gene flow from cultivated potato to wild potato is considered unlikely due to very limited geographic overlap of cultivated and wild-potatoes in the U.S., agricultural practices, and biological characteristics of potatoes in general (OECD, 1997).

**Deregulated Events with Down Regulated PPO**

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

- E12, F10, J3, and others (10 events total), 13-022-01p[1];
- W8, 14-093-01p[2];
- V11, 15-140-01p[3]; and
- X17 and Y9, 16-064-01p[4].

**No Plant Pest Risk or Increased Weed Potential**

USDA-APHIS has previously concluded there is no reason to believe that changes to the plant genome generated by the gene editing process of site-directed nucleases would generate a plant pest (Table 2).

**Table 2. USDA-APHIS References Regarding Site-Directed Nucleases**

| Reference | Date | Site-directed nuclease | Crop |
|---|---|---|---|
| Gregoire to Dow AgroSciences | 8-Mar-12 | zinc-finger nuclease | Corn |
| Firko to Cellectis Plant Sciences | 28-Aug-14 | TALENs | Potato |
| Firko to Cellectis Plant Sciences | 5-May-15 | TALENs | Soybean |
| Firko to Cellectis Plant Sciences | 20-May-15 | TALENs | Soybean |
| Firko to Calyxt | 1-Feb-16 | TALENs | Wheat |
| Firko to Calyxt | 15-Sep-16 | TALENs | Potato |

[1] https://www.aphis.usda.gov/brs/aphisdocs/13_02201p_ppra.pdf
[2] https://www.aphis.usda.gov/brs/aphisdocs/14_09301p_fpra.pdf
[3] https://www.aphis.usda.gov/brs/aphisdocs/15_14001p_det.pdf
[4] https://www.aphis.usda.gov/brs/aphisdocs/16_06401p_det_pprsa.pdf

In the plant pest risk assessments of Simplot's deregulated events with PPO down-regulation, 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[5]:

- The events pose no more of a plant pest risk than their respective parental varieties or other conventional potato 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 taxa compared to the nontransgenic recipient or other varieties of the crop commonly grown; and
- The deregulated potato events are unlikely to persist as a troublesome weed or to have an impact on current weed management practices.

Therefore, the proposed low PPO5 potatoes are unlikely to change the plant pest risk or increased weed potential compared to conventional potatoes.

**No Plant Pest Sequences Remain in Low PPO5 Potatoes**

Targeted *Ppo5* knockout potatoes are generated through transient expression of the TALEN cassettes. The TALEN, FMV promoter, overdrive, pVS1, and *ipt* sequences in the binary vector are derived from plant pest sequences (*Xanthomonas spp.*, fig mosaic virus, *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*) (Table 1) as designated in 7 CFR 340.2. However, these sequences are not involved in pathogenicity and do not express proteins that would result in infection or pathogenicity of low PPO5 potato events. Importantly, the final low PPO5 potato product is like a null segregant in that it does not contain these sequences.

APHIS has previously made determinations that genetically modified plants transformed with TALENs that are segregated away are not regulated articles, for example, null segregants of potatoes, soybean and wheat (Table 2). In addition, APHIS has previously determined the following are not regulated articles:

- Null segregant of sorghum transformed via *Agrobacterium* (Gregoire to University of Nebraska, June 6, 2012); and
- Null segregant of corn generated with Meganucleases (Firko to Agrivida, Nov. 12, 2015).

[5] https://www.aphis.usda.gov/brs/aphisdocs/13_02201p_ppra.pdf

**Conclusions**

Simplot Plant Sciences has developed a method to use *Agrobacterium* to transiently deliver site-directed nucleases into plant cells and select for regenerated plantlets with reduced PPO5 and no inserted sequence from the vector. The end product is like a null segregant because no genetic material used to create the targeted deletion is present. Low PPO5 potatoes contain no plant pest sequences, and the phenotype is highly unlikely to result in increased weediness or plant pest potential.

Prior to further investigation of both the method and product development of low PPO5 potatoes, Simplot requests confirmation from Biotechnology Regulatory Services that Simplot's low PPO5 potatoes (*Solanum tuberosum,* L.) do not meet the definition of a regulated article under 7 CFR Part 340.

We eagerly await your response to our enquiry.

Sincerely,

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