UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

SUBJECT: Review of the Application for an Experimental Use Permit for Gen 3 Potatoes expressing transgenic R-proteins BLB2, AMR3 and VNT1, PVY Coat Protein Hairpin RNA and inert ingredient StmALS and associated FFDCA Petitions for the Temporary Exemption from a Tolerance for AMR3 and BLB2, as well as FFDCA Petition for the Exemption from a Tolerance for StmALS.

Experimental Use Permit for Gen 3 Potatoes:

EPA File Symbols	8917-EUP-G
Pesticide Petition No.	0G8830
PC code:	006660, 006661, 006662, 006355, 817309
E-submission package ID No.	1048674
Action code case No.	00135196
MRID Nos.	51073802, 51073803, 51073805, 51211400, 51211403, 51211407, 51529201, 51976802, 52055401, 52055402, 52055404, 52055405.

Petition for the Exemption from a Tolerance for StmALS:

EPA File Symbols	IN-11411
PC code:	817309
E-submission package ID No.	1048676
Action code case No.	00135455
MRID Nos.	51073808, 51211403, 51211406, 51211407, 51529202, 51976802, 51211407, 51785602, 52055403, 52055404, 52086800.

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The conclusions conveyed in this assessment were developed in full compliance with EPA Scientific Integrity Policy for Transparent and Objective Science, and EPA Scientific Integrity Program's Approaches for Expressing and Resolving Differing Scientific Opinions. The full text of EPA Scientific Integrity Policy for Transparent and Objective Science, as updated and approved by the Scientific Integrity Committee and EPA Science Advisor can be found here: https://www.epa.gov/sites/default/files/2014-02/documents/scientific_integrity_policy_2012.pdf. The full text of the EPA Scientific Integrity Program's Approaches for Expressing and Resolving Differing Scientific Opinions can be found here: https://www.epa.gov/scientific-integrity/approaches-expressing-and-resolving-differing-scientific-opinions

I. Executive Summary

Regulatory History

J.R. Simplot Company requested an Experimental Use Permit for Gen 3 potatoes expressing transgenic active ingredients BLB2, AMR3 and VNT1, PVY Coat Protein Hairpin RNA (PVY CP hpRNA) and the new inert ingredient modified acetolactate synthase protein (StmALS) from potato.

A request for temporary exemption from the requirement of a tolerance for residues of BLB2 and AMR3 proteins and the genetic material necessary for their production, the *Rpi-blb2* and *Rpi-amr3* genes, respectively, in potato (Pesticide Petition 0G8830), and a request for permanent exemption from the requirement of tolerance for residues of StmALS in potato (IN-11411) were submitted (MRIDs 51073802 and 51211406). An exemption from the requirement of a tolerance has previously been granted for the *Rpi-vnt1* gene that expresses the VNT1 protein (40 CFR §174.534). Similarly, EPA has established an exemption from the requirement of a tolerance for residues of nucleic acids that are part of a plant-incorporated protectant (40 CFR § 174.507). Therefore, EPA has not conducted an assessment under FFDCA for PVY CP hpRNA and the Agency was not petitioned to establish a tolerance exemption for PVY CP hpRNA.

Product characterization

BLB2, AMR3, and PVY CP hpRNA are new active ingredients. BLB2 and AMR3 are R-genes from *Solanum bulbocastanum* and *Solanum americanum*, respectively. These ingredients are intended to provide resistance to *Phytophthora infestans* infections. *Phytophthora infestans* is an oomycete that causes a serious potato (and tomato) disease known as late blight.

PVY CP hpRNA is derived from the sequence of the gene encoding the Potato Virus Y Coat Protein, and is designed to target Potato Virus Y. Potato Virus Y can cause the development of necrotic spots on potato tubers and foliage, and decrease in yield.

Gen 3 potatoes also carry an inert ingredient, the modified acetolactate synthase protein (StmALS) from *Solanum tuberosum*. Acetolactate synthase is an enzyme catalyzing the first step in the synthesis of branched amino acids; the modifications introduced allow the enzyme to be resistant to certain herbicides. This feature allows the use of StmALS as a selective marker for transformation events.

Finally, Gen 3 potatoes carry two non-pesticidal traits coding for hairpin interfering RNAs. These traits target two endogenous potato genes, polyphenol oxidase (Ppo) to reduce black spot and vacuolar invertase (Inv) to lower reducing sugars.

TABLE 1. Plant-Incorporated Protectants in Gen 3 Potatoes and Their Target Pests.

COMMON NAME	PROTEIN	PC CODE	TARGET PEST
VNT1	VNT1	006355	*Phytophthora infestans*
BLB2	BLB2	006661	*Phytophthora infestans*
AMR3	AMR3	006660	*Phytophthora infestans*
PVY CP hpRNA gene	None	006662	Potato Virus Y
StmALS	StmALS	817309	None – selectable marker

Mammalian Toxicity

The studies addressing mammalian toxicity for BLB2 and AMR3 include bioinformatics studies of toxicity and allergenicity potential of BLB2 and AMR3. The applicant submitted waiver requests for Acute Toxicity studies and studies related to allergenicity (glycosylation and stability to heat and gastric proteases), which EPA waived based on low levels of expression, the history of safe use for BLB2, AMR3, and multiple homologous R-proteins, and a non-toxic mode of action, as well as the lack of homology to known toxins and allergens in bioinformatics studies.

The studies addressing mammalian toxicity for StmALS include bioinformatics study of toxicity and allergenicity potential of StmALS, stability to heat and gastric proteases. In support of the waivers for Acute Toxicity studies, the applicant cited the lack of homology to known toxins and allergens, susceptibility to deactivation by heat and by gastric proteases, as well as the high percent identity (99.7%) between StmALS and native *Solanum tuberosum* StALS which has a history of safe use, and the ubiquitous presence of acetolactate synthase genes in edible crops with a history of safe use.

Based on the results of the bioinformatics studies, origins of the traits, mechanism of action, a history of safe use arguments, as well as, for StmALS, the susceptibility to heat treatment and digestion by gastric proteases, the EPA concludes that BLB2, AMR3 and StmALS do not pose any risk of toxicity to humans.

The assessment of mammalian toxicity of PVY Coat Protein hairpin RNA was based on the molecular characterization data, expression data and human health rationale. Further, the existing tolerance exemption for residues of nucleic acids that are part of a plant-incorporated protectant (40 CFR § 174.507) is applicable to the PVY Coat Protein hairpin expressed in the Gen 3 potato, indicating a negligible risk to human or livestock that consume potato products.

Allergenicity

Since BLB2, AMR3 and StmALS are proteins, their potential for food allergenicity was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a "weight-of-evidence" approach when considering the allergenic potential for a PIP protein and bases its conclusions upon the following factors: the source of the trait, the amino acid sequence compared with known allergens, and the biochemical properties of the protein, including *in vitro* digestibility in simulated gastric fluid (SGF) and glycosylation. This is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants."

The Agency's allergenicity assessment for the BLB2 and AMR3 follows:

1. **Source of the traits**. The source of the BLB2 protein is the wild species *Solanum bulbocastanum*. *S. bulbocastanum* is not typically consumed. However, two recently released, conventionally bred varieties, Toluca and Bionica, contain the *Rpi-blb2* gene. These varieties are cultivated on a small scale, primarily in Europe (Haverkort et al., 2009). There are no reports of these varieties harboring allergenic proteins.

   The source of AMR3 protein is *Solanum americanum.* Both leaves and berries of *S. americanum* are consumed by humans. The leaves are boiled, drained, and eaten in Africa and Asia (Edmonds and Chweya, 1997; Yuan *et al*., 2018). There are no reports of allergies caused by *S. americanum*.

2. **Amino acid sequence.** A comparison of the amino acid sequences of BLB2 and AMR3 with known allergens showed no sequence identity at the level of eight contiguous amino acid residues (length based on the expected size of IgE epitopes (Chatchatee et al., 2001; Shin et al., 1998)), and no sequence identity over 35% in a sliding 80 aa window.

3. **Digestibility.** Data was not submitted.

4. **Glycosylation.** Data was not submitted.

5. **Conclusion.** EPA concluded that the potential for BLB2 and AMR3 to be a food allergen is minimal.

The Agency's allergenicity assessment for the StmALS protein follows:

1. **Source of the traits:** The StmALS is 99.7% identical to the native *S. tuberosum* acetolactate synthase (StALS) with only two amino acid changes. *S. tuberosum* is not known to be a source of allergenic proteins.

2. **Amino acid sequence.** A comparison of the amino acid sequences of StmALS known allergens showed no sequence similarity or identity at the level of eight contiguous amino acid residues (length based on the expected size of IgE epitopes (Chatchatee et

al., 2001; Shin et al., 1998)), and no sequence identity over 35% in a sliding 80 aa window.

2. **Digestibility.** The recombinant StmALS protein was digested rapidly in simulated gastric fluid containing pepsin, the enzyme produced by the stomach that digests proteins so they can be absorbed as nutrients into the body via the small intestine. It was also rapidly digested by pancreatin, which is a mixture of several digestive enzymes produced by the pancreas. The rapid degradation of StmALS in the simulated gastric environment indicated that the intact protein will not pass from the stomach into the intestinal lumen, where sensitization of the immune system to food allergens occurs.

3. **Heat stability.** The activity of the recombinant StmALS was measured following heating using colorimetric assay. StmALS activity completely deteriorates at temperatures above 56 °C. In a separate experiment, the StmALS protein was visualized using immunoblotting following thermal treatment. A soluble StmALS protein band was not detected by SDS-PAGE following heat treatment at 90 °C for 60 minutes when samples were centrifugated. Thus, StmALS protein is heat-labile. Potential conformational IgE epitopes will likely denature after thermal preparation, abolishing capacity for IgE binding and allergenicity of StmALS.

4. **Glycosylation.** Current scientific knowledge suggests that protein glycosylation may contribute to protein stability and enhance its allergenic potential (Pedrosa et al., 2000; Wormald et al., 1999; Shreffler et al., 2006). StmALS protein expressed in *S. tuberosum* was not found to be glycosylated.

5. **Conclusion.** EPA concluded that the potential for StmALS to be a food allergen is minimal.

II. Recommendation

The molecular characterization and human health data support the finding that there is reasonable certainty that no harm will result from the aggregate exposure to the U.S. population, including infants and children to the BLB2, AMR3 and StmALS proteins and the genetic material necessary for their production in potato event BG25. This includes all the anticipated dietary exposure and all other exposures for which there is reliable information.

Therefore, the data submitted for potato event BG25 are acceptable to support the temporary petition for an exemption from the requirement of a tolerance for residues of BLB2 and AMR3 proteins in/on the food and feed commodities of potato, as well as the permanent petition for an exemption from the requirement of a tolerance for residues of StmALS protein in/on the food and feed commodities of potato.

Based on the molecular characterization data, expression data and human health rationale for the PVY Coat Protein hairpin RNA expressed in the Gen 3 potato, EPA has determined that no unreasonable adverse effects to humans are expected from the PVY Coat Protein hairpin and the

genetic material necessary for its production in the Gen 3 potato during the proposed EUP. Further, the existing tolerance exemption for residues of nucleic acids that are part of a plant-incorporated protectant (40 CFR § 174.507) is applicable to the PVY Coat Protein hairpin expressed in the Gen 3 potato, indicating a negligible risk to human or livestock that consume potato products.

An exemption from the requirement of a tolerance has previously been granted for the *Rpi-vnt1* gene that expresses the VNT1 protein (40 CFR §174.534). EPA's analysis confirms that this established exemption is applicable to the *Rpi-vnt1* gene and VNT1 protein expressed in Gen 3 potatoes.

It is recommended that the following shortcoming be addressed with any Section 3 applications submitted for event BG25 potatoes:

- Quantification studies for PVY CP hairpin RNA to determine Certified Limits.

DATA REVIEW RECORD

III. Product Characterization

A. Background

J.R. Simplot Company is seeking an Experimental Use Permit for Gen 3 potatoes expressing transgenic R-proteins BLB2, AMR3 and VNT1, PVY Coat Protein Hairpin RNA, and the inert ingredient modified acetolactate synthase protein (StmALS) from potato. Petition for a temporary exemption from the requirement of a tolerance for residues of BLB2 and AMR3 proteins (EPA file symbol 0G8830), and a Petition for permanent exemption from the requirement of a tolerance for residues of StmALS (EPA File Symbol IN-11411) were also submitted. An exemption from the requirement of a tolerance exists for the *Rpi-vnt1* gene that expresses the VNT1 protein (40 CFR §174.534), therefore the Agency was not Petitioned to establish a new tolerance for the VNT1 protein. Similarly, EPA has established an exemption from the requirement of a tolerance for residues of nucleic acids that are part of a plant-incorporated protectant (40 CFR § 174.507, redesignated from § 174.475, effective April 25, 2007). Therefore, EPA has not conducted an assessment under FFDCA for PVY-CP hairpin RNA gene and the Agency was not Petitioned to establish a tolerance exemption for PVY-CP hairpin RNA gene.

Gen 3 potatoes were developed by the J.R. Simplot Company using biotechnology to introduce traits for improved disease protection. The following traits were introduced in Gen 3 potatoes through *Agrobacterium*-mediated transformation with plasmid pSIM4363:
- protection against late blight infection caused by *Phytophthora infestans* from three resistance proteins: BLB2, AMR3 and VNT1;
- protection against Potato virus Y (PVY) infection through small interfering RNA (siRNA) from PVY Coat Protein Hairpin RNA.
- an inert ingredient, the modified acetolactate synthase protein (StmALS) from potato; this protein provides herbicide resistance that is used for selection of transformation events.
- two non-pesticidal traits coding for hairpin interfering RNAs; these traits target two potato genes, polyphenol oxidase (Ppo) for reduced black spot and vacuolar invertase (Inv) for lower reducing sugars.

R-proteins (such as BLB2, AMR3 and VNT1) function as receptors, directly or indirectly recognizing specific pathogen-secreted proteins that suppress the plant's initial immune response (effectors). The R-proteins encoded by the *Rpi-blb2* (BLB2), *Rpi-amr3* (AMR3), and *Rpi-vnt1* (VNT1) genes recognize specific pathogen-secreted effector proteins (Avr-blb2, Avr-amr3, and Avr-vnt1, respectively) and initiate a signaling pathway leading to a plant hypersensitive response (Dodds and Rathjen, 2010; Spoel and Dong, 2012; Stefanczyk et al., 2017). The plant hypersensitive response is a form of programmed cell death, in which plant cells around the area of pathogen penetration are killed to prevent spread of the infection (Morel and Dangl, 1997). R-proteins do not act on the pathogen but confer disease protection by activating the plant's immune response. The presence of three R-proteins in the Gen 3 potato that recognize different *P. infestans* effectors is expected to contribute to the durability of the late blight protection trait.

The PVY down regulation cassette was designed to target the coat protein region of the PVY RNA genome via RNA interference. The cassette contains part of the PVY coat protein (PVY-CP) sequence from strain PVYN arranged into an inverted repeat that encodes a dsRNA hairpin when transcribed. The inverted repeat is designed to generate a dsRNA hairpin with a 237 bp intervening spacer sequence (Figure 1). The spacer sequence is derived from the granule-bound starch synthase gene from the Ranger Russet potato variety. The PVY coat protein DNA is oriented as an inverted repeat in the Gen 3 potato which results in the production of dsRNA but not production of a protein. The mechanism of action is based on a natural defense pathway evolved in plants against viruses using post-transcriptional gene silencing and siRNAs. As the siRNAs produced from the dsRNA hairpin are sequence-specific to the PVY coat protein, they then target the viral RNA genome of PVY for degradation and block viral replication, thus conferring PVY protection.

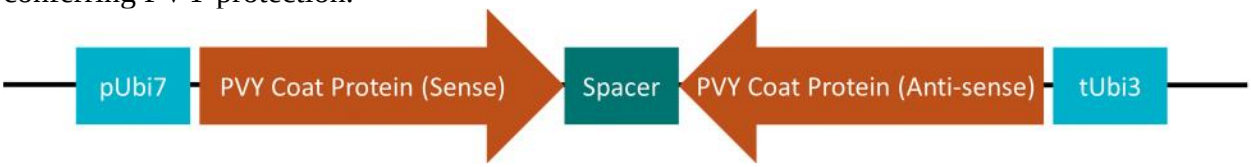

Figure 1. PVY Coat Protein Inverted Repeat from pSIM4363

The PVY-CP inverted repeat from pSIM4363 is designed with a 5´ polyubiquitin promoter (pUbi7), followed by 522 bp from the PVY coat protein sequence, a 237 bp spacer sequence that forms a loop in the dsRNA hairpin structure, 522 bp from the PVY coat protein sequence in the antisense orientation, and a 3´ polyubiquitin terminator (tUbi3).

A modified acetolactate synthase (StmALS) conferring herbicide tolerance was introduced into potato as an inert ingredient to be used for screening positive transformation events. ALS catalyzes the first common step in the synthesis of the branched chain amino acids, isoleucine, leucine, and valine. This enzyme is targeted by such herbicides as sulfonylureas and imidazolinone herbicides. StmALS acts as a selectable marker via tolerance to these herbicides. StmALS differs from the native potato ALS (StALS) by two amino acid substitutions: the tryptophan residue at 563 changed to leucine, and the serine residue at 642 changed to isoleucine (W563L, S642I). These amino acid substitutions interfere with herbicide binding to StmALS, therefore neutralizing herbicide's inhibitory effect.

B. The Transformation System

All traits in Gen 3 potatoes were introduced through Agrobacterium-mediated transformation with plasmid pSIM4363 (Figure 2).

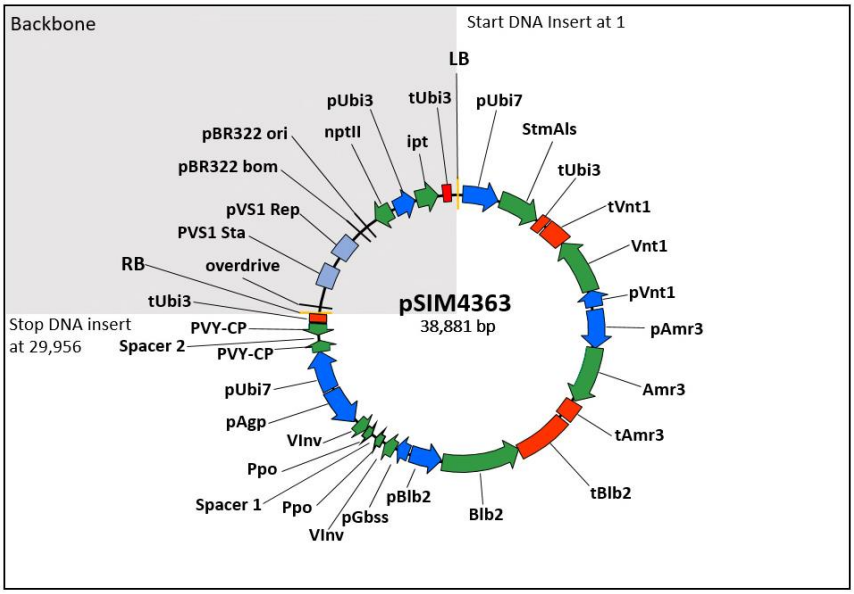

Figure 2. Plasmid Map of pSIM4363.

The T-DNA is 29,956 bp in length and consists of six cassettes designed to express RNA inverted repeats or gene coding sequences. The entire T-DNA is flanked by left border and right border regions necessary for Agrobacterium-mediated insertion into the potato genome.

Three of the cassettes in pSIM4363 are designed to introduce three different potato resistance genes (R-genes): *Rpi-vnt1* from *S. venturi*, *Rpi-amr3* from *S. americanum*, and *Rpi-blb2* from *S. bulbocastanum*. Transcription of each R-gene is regulated by the native promoter and terminator sequences for that gene.

Two of the cassettes in the pSIM4363 T-DNA are designed as inverted repeats for the down regulation of gene expression through the RNA interference (RNAi) pathway.

- The first down regulation cassette targets two potato genes, polyphenol oxidase (Ppo) for reduced black spot and vacuolar invertase (Inv) for lower reducing sugars. Transcription of the Ppo/Inv inverted repeat is regulated by two inward facing promoters, the granule-bound starch synthase gene promoter (pGbss) and the ADP glucose pyrophosphorylase gene promoter (pAgp), both from potato. Down regulation of PPO and INV proteins is not claimed to result in pest mitigation, but is intended to improve tuber quality. PPO and INV are not considered plant-incorporated protectants (PIPs).
- The second down regulation cassette targets the gene encoding the coat protein of Potato virus Y (PVY-CP), for conferring PVY protection to the plant. Transcription of the PVY-CP inverted repeat is regulated by a polyubiquitin promoter (pUbi7) and terminator (tUbi3), both from potato.

The final cassette in the pSIM4363 T-DNA was designed to express a modified potato acetolactate synthase gene (StmAls) for use as a selection marker during tissue culture selection. The gene is controlled by promoter pUbi7 and terminator tUbi3 from potato.

The pUbi7 promoter used for expression of StmALS and PVY-CP hairpin RNA contains a 569 bp intron in the 5' untranslated region and the ubiquitin coding sequence (Garbarino et al., 1995). StmALS is expressed as a fusion with ubiquitin, which is expected to be rapidly cut by endogenous ubiquitin-specific proteases. The resulting StmALS contains an N-terminal Chloroplast-Targeting Peptide (CTP) and is further processed via removal of CTP following localization to plastids. PVY-CP hairpin RNA also contains ubiquitin coding sequence, but because it enters the RNAi-processing pathway, it is not translated. Instead, siRNAs are produced from its sequence.

C. Characterization of the DNA Inserted in the Plant

T-DNA sequence and insertion site were determined using Illumina sequencing after targeted capture with probes designed to span the whole length of the pSIM4363 plasmid used for transformation of BG25 potato. The insert in BG25 contains a nearly full-length T-DNA, missing 330 bp from the 5' end of the annotated left border element (with a partial deletion of the sequences in the upstream region of the pUbi7 promoter directing the transcription of the StmALS gene) and 34 bp from the 3' end of the annotated right border element.

The sequences corresponding to the insertion site in BG25 and parent non-transformed strain were cloned and sequenced. The insertion site had homology on chromosome XII of the potato reference genome and contained a 55 bp deletion at the site of insertion, compared to parent strain. No annotated open reading frames (ORFs) were interrupted by the insert. The closest annotated ORF ("chilling-responsive protein") is found at approximately 3 kb distance from the insertion site. No novel ORFs fused to PIPs were created at the junction sites.

The sequencing data also confirmed the absence of the pSIM4363 backbone in BG25 potato. This was further confirmed using amplification of a series of 6 locations in the backbone of pSIM4363 (Figure 3). Finally, the absence of the plasmid backbone is confirmed by phenotypic screening of the transformed potato for the lack of ipt expression (a gene located in the pSIM4363 backbone). Altogether, the absence of backbone is well supported by the data obtained the three methods described above.

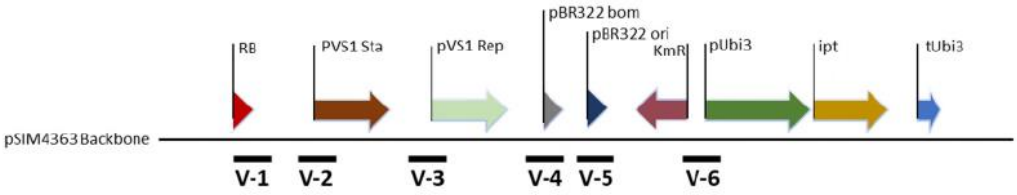

Figure 3. Plasmid Backbone Detection PCR Assays

The locus and copy number of the insert were determined by ddPCR. Eight amplification sites unique for the insert (located at the junction of the genes within T-DNA) and spread over the length of the T-DNA were assayed (Figure 4). The locus and copy number for all of the tested sites was determined to be 1 per the 4n potato genome.

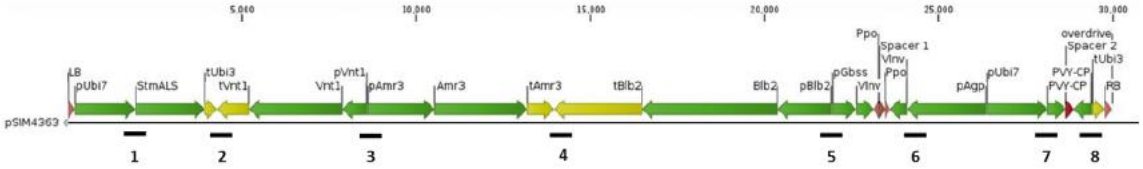

Figure 4. Regions of the pSIM4363 T-DNA Assayed by ddPCR
Assays for targets labeled 1-8 were designed to amplify junction regions in pSIM4363 for quantification relative to the reference genes.

Potatoes are propagated vegetatively and in vegetative propagation the vegetative parts of the plant multiply by mitosis giving rise to a new plant which is a true copy of the parent plant (i.e., no fusion of gametes, genetic recombination, or meiotic divisions). Because of this form of reproduction, studies examining Mendelian segregation of the insert-encoded traits and trait stability over several generations were not required for the Gen 3 potato.

D. Plant-incorporated Protectant Expression

The detection of BLB2 and AMR3 was attempted using immunoblot methods. Polyclonal antibodies were developed using peptide sequences near the C-terminal end from each of the three R-proteins. For AMR3 detection, the limit of quantitation was established at 250 ppb FW in leaf and 500 ppb FW in tuber tissues, using samples spiked with *E. coli* - expressed AMR3. The AMR3 protein was undetectable in BG25 leaf and tuber samples. The limit of quantitation for BLB2 was established at 250 ppb FW in leaf and 100 ppb FW in tuber tissues, using samples spiked with *E. coli* - expressed BLB2. The BLB2 protein was also undetectable in BG25 leaf and tuber samples. The literature data supports that R-proteins are tightly regulated and expressed at low levels in plants to reduce metabolic costs associated with aberrant R-protein activation that could cause unnecessary cell death not associated with pathogen infection (Marone et al., 2013). This suggests that the R-proteins are indeed expressed at levels below the LOQs, and it is not a consequence of inadequate experimental conditions. Moreover, the authors were able to demonstrate *amr3* and *blb2* transcript expression in leaf and tuber of BG25 using RT-qPCR (MRIDs 51976805, 51976806), but the *blb2* and *amr3* transcripts could not be quantified in absolute units.

To quantify StmALS, a polyclonal antibody was developed using a peptide sequence from StmALS to detect StmALS in leaf and tuber tissues. This antibody was directed against the W563L mutation in the ALS protein. As a result, this antibody recognizes the potato StmALS, and not the native potato ALS. The StmALS protein was detected by immunoblotting and quantified using densitometry in BG25 leaf and tuber samples. The average StmALS protein expression level was 1,830 ppb FW in leaf (range 780-6,010 ppb) and 420 ppb FW in tubers (range 200-570 ppb).

The company has not developed a method to quantify PVY-CP hairpin RNA content. Instead, the company has estimated the content of PVY-CP siRNAs at 0.042 µg/g FW, based on the scientific literature.

E. Supporting Data

The submitted product characterization studies to support the registrant's application for an Experimental Use Permit for Gen 3 Potatoes expressing transgenic R-proteins BLB2, AMR3, VNT1, PVY Coat Protein Hairpin RNA and StmALS and associated request for temporary exemption from the requirement of a tolerance for residues of AMR3 and BLB2 are summarized with their classifications in Table 2.

The submitted product characterization studies to support the registrant's request for permanent exemption from the requirement of a tolerance for residues of StmALS are summarized with their classifications in Table 3.

The Agency individually reviewed the submitted studies in Data Evaluation Reports (DERs).

The information provided is sufficient to support the product characterization and manufacturing process for the Experimental Use Permit and FFDCA Petition for the Temporary Exemption from a Tolerance for Gen 3 Potatoes expressing these active ingredients, as well as the FFDCA Petition for the Exemption from a Tolerance for StmALS.

Table 2. Summary of Product Identity, Manufacturing Process, and Analytical Methods Data Submitted in Support of the Experimental Use Permit of Gen 3 Potatoes Containing VNT1, BLB2, AMR3, PVY-CP hairpin gene, and StmALS

STUDY TYPE	RESULT	MRID NO.
UPDATED: Experimental Use Permit Application—8917-EUP-G: Gen 3 Potatoes	Part of this MRID contains data on product identity for Gen 3 potatoes, including protein or DNA sequences (for hairpin RNA) of all the pesticidal traits, source of the genes, map of the vector, regulatory sequences, selection markers, target organisms, and mode of action. The classification below is relevant to this part only. **Classification: Acceptable**	51211403
Data Requirements and Waivers	This is a table of waivers for product characterization, residue and toxicology requirements for BLB2, AMR3 and VNT1. Scientific rationales in support of PVY hairpin RNA, BLB2, AMR3 and VNT1 safety are found in MRID 51211403. **Classification: Acceptable**	51211407
Sequence Characterization of the Insert in BG25	Mate pair libraries and multiplexed targeted sequencing were used in conjunction with NGS data, Sanger sequencing, PCR and droplet digital PCR (ddPCR) to determine insertion site structure, flanking sequence, locus and copy number of amplicons, and absence of backbone in BG25.	52055405 51211400

Table 2. Summary of Product Identity, Manufacturing Process, and Analytical Methods Data Submitted in Support of the Experimental Use Permit of Gen 3 Potatoes Containing VNT1, BLB2, AMR3, PVY-CP hairpin gene, and StmALS

STUDY TYPE	RESULT	MRID NO.
	In MRID 51211400, the absence of pSIM4363 backbone in experimental plants is further confirmed by phenotypic screening against events containing a pSIM4363 backbone element *ipt*. **Classification: Acceptable**	
Allergen and Toxin Evaluation of Open Reading Frames in BG25	A bioinformatics-based analysis was provided describing the identification of open reading frames in and around the T-DNA insertion site within the transformed potato line BG25. Two short ORFs spanning the junctions of the insert in BG25 were identified. These ORFs do not create fusion with any PIPs, are non-allergenic, and predicted to be non-toxic. They are also unlikely to be transcribed. Two previously unreported fusion ORFs were identified within the insert itself. One is polyubiquitin-StmALS fusion, the other polyubiquitin-PVY-CP-derived ORF fusion. Neither fusion ORF indicates a concern regarding toxicity or allergenicity, and neither is likely to result in a stable fusion protein. Thus, identified fusion ORFs do not present a concern. **Classification: Acceptable**	52055404 52086800 52086801
AMR3 Protein and *Rpi-amr3* Transcript Expression in BG25	The limit of quantitation (LOQ) for AMR3 was established at 250 parts per billion (ppb) in leaf and 500 ppb in tuber tissues. The AMR3 protein was undetectable in BG25 leaf and tuber samples. Therefore, Rpi-amr3 transcript expression was measured by RT-qPCR to demonstrate the presence of the *Rpi-amr3* gene in BG25. **Classification: Acceptable**	52055401
BLB2 Protein and *Rpi-blb2* Transcript Expression in BG25	The limit of quantitation (LOQ) for BLB2 was established at 250 parts per billion (ppb) FW in leaf and 100 ppb FW in tuber tissues. The BLB2 protein was undetectable in BG25 leaf and tuber samples. Therefore, Rpi-blb2 transcript expression was measured by RT-qPCR to demonstrate the presence of the *Rpi-blb2* gene in BG25. **Classification: Acceptable**	52055402

Table 3. Summary of Product Identity, Manufacturing Process, and Analytical Methods Data Submitted in Support of the FFDCA Petition for the Exemption from a Tolerance for StmALS		
STUDY TYPE	**RESULT**	**MRID NO.**
UPDATED: Experimental Use Permit Application—8917-EUP-G: Gen 3 Potatoes	Part of this MRID contains data on product identity for StmALS, including protein sequence, source of the gene, and mode of action. The classification below is relevant to this part only. **Classification: Acceptable**	51211403
StmALS Protein Expression in BG25	The average StmALS protein expression level was 1,830 ppb FW in leaf (range 780-6,010 ppb) and 420 ppb FW in tubers (range 200-570 ppb). **Classification: Acceptable**	51976802

IV. Hazard Analysis

The submitted mammalian toxicity studies to support the registrant's application for a FIFRA Section 5 Experimental Use Permit for PVY Coat Protein Hairpin RNA, and transgenic R-proteins BLB2, AMR3, and VNT1 expressed in Gen 3 Potatoes are summarized with their classifications in Table 4. Scientific rationales were submitted in support of oral, dermal, inhalation, and ocular pathways. These studies were also submitted in support of the associated request for a temporary exemption from the requirement of a tolerance for residues of AMR3 and BLB2 proteins.

The submitted mammalian toxicity studies to support the registrant's application for the petition for the exemption from a tolerance for residues of the StmALS protein are summarized with their classifications in Table 5. Scientific rationales for StmALS were submitted in support of the oral, dermal, inhalation, and ocular pathways.

Information from the scientific rationales and studies is included in the section below, and Data Evaluation Records (DERs) of the scientific rationales and studies are attached.

The information provided is sufficient to support the human health risk assessment for the FIFRA Section 5 Experimental Use Permit application for these PIPs. It is also sufficient to support the FFDCA Petitions for the Temporary Exemption from a Tolerance for AMR3 and BLB2, and FFDCA Petition for the Exemption from a Tolerance for StmALS. Further testing at higher toxicological tiers is not required.

Table 4. Summary of Mammalian Toxicity Data Submitted in Support of the Section 3 Registration of Gen 3 Potatoes containing BLB2, AMR3 and VNT1, PVY Coat Protein Hairpin RNA and an inert ingredient StmALS, and FFDCA Petitions for the Temporary Exemption from a Tolerance for AMR3 and BLB2.

STUDY TYPE	RESULT	MRID NO.
870.1100 Acute oral toxicity 870.1200 Acute dermal toxicity 870.1300 Acute inhalation toxicity 870.2400 Acute eye irritation 870.2500 Primary dermal irritation	Data waiver. -R-proteins affect the plant by activating a localized plant hypersensitive response. R-proteins are not directly toxic to the late blight pathogen. R-proteins are not known to be acutely toxic. - The BLB2 protein, encoded by Rpi-*blb2*, is present in two potato varieties (Toluca and Bionica) cultivated for food use in Europe. - The Rpi-*amr3* gene is from the wild *Solanum* species, *Solanum americanum*, which is consumed by humans and used both for food and medicinally. -No similarity to known toxins or allergens were identified in bioinformatics analysis of BLB2 and AMR3. -The expression of R-proteins is maintained at a low level in plants. In potato plants transformed with pSIM4363, BLB2 and AMR3 expression was below the LOQ. -The dietary exposure to humans and livestock is low. **Classification: Acceptable** **Toxicity category:** EPA Toxicity Category IV	51073803 51211403 51073802
Allergen and Toxin Evaluation of BLB2, AMR3, and VNT1	The allergen and toxin potential of BLB2 and AMR3 was assessed using bioinformatic techniques. There were no matches between sequences in the AllergenOnline database and BLB2 or AMR3in any of the searches (i.e.,80-mer, or 8-mer). Similarly, no homology to actual toxins was identified for BLB2 or AMR3 proteins. **Classification: Acceptable**	51073805 51529201 52055404 52086801

Table 5. Summary of Mammalian Toxicity Data Submitted in Support of the FFDCA Petition for the Exemption from a Tolerance for StmALS.

STUDY TYPE	RESULT	MRID NO.
870.1100 Acute oral toxicity 870.1200 Acute dermal toxicity 870.1300 Acute inhalation toxicity 870.2400 Acute eye irritation	Data waiver. - The ubiquitous presence of acetolactate synthase genes in edible crops with a history of safe use, -StmALS does not have sequence similarity with known toxins or allergens. -The StmALS is 99.7% identical to the native potato ALS, which has a long history of safe use.	51211407 51211403 51211406

Table 5. Summary of Mammalian Toxicity Data Submitted in Support of the FFDCA Petition for the Exemption from a Tolerance for StmALS.

STUDY TYPE	RESULT	MRID NO.
870.2500 Primary dermal irritation	-Similar amino acid substitutions introduced into StmALS are also found in commonly consumed crops such as maize, wheat, and rice, which are safely consumed by humans. **Classification: Acceptable** **Toxicity category:** EPA Toxicity Category IV	
UPDATED: Data Requirements and Waivers	This is a table of waivers for product characterization, residue and toxicology requirements for StmALS. Scientific rationales in support of StmALS safety are found in MRID 51211403. **Classification: Acceptable**	51211407
Simulated Gastric and Intestinal Digestion and Heat Lability of Potato Modified Acetolactate Synthase (StmALS)	StmALS was hydrolyzed in less than 30 sec in both SGF and SIF. StmALS activity was heat labile above 46 °C. StmALS activity began to decrease above 46 °C and no measurable StmALS activity was observed at temperatures above 56 °C. A soluble StmALS protein band was not detected by SDS-PAGE following heat treatment at 90 °C. **Classification: Acceptable**	52055403 52086800
Glycosylation Status of Modified Acetolactate Synthase (StmALS) in pSIM4363 Transformed Potatoes	Protein was extracted from tubers of potato plants transformed with pSIM4363, which express both endogenous (unmodified) StALS and modified StmALS. SDS-PAGE separation and a Glycoprotein Staining Kit were used to detect any glycosylated proteins. StmALS glycosylation was not detected in this study. **Classification: Acceptable**	51785602
Bioinformatic Assessment of Potential Allergenicity and Toxicity for Potato Acetolactate Synthase	The results of bioinformatics search showed that neither StmALS or native StALS were homologous to known allergens in either 80-mer 35% identity or 8-mer exact match searches. Similarly, no homology to actual toxins was identified for either the StmALS or native StALS proteins. **Classification: Acceptable**	51073808 51529202 52055404 52086801

A. BLB2 and AMR3

1. Toxicological profile

Mammalian toxicity was examined via a "weight of evidence" approach using mode of action, expression levels, history of safe use, and bioinformatics. Based on the analysis below, BLB2

and AMR3 proteins represent a negligible risk to human or livestock that consume potato products.

Mode of action

R-proteins (such as BLB2 and AMR3) confer protection against pathogens by directly or indirectly recognizing pathogen-secreted effector proteins (Jones and Dangl, 2006). BLB2 recognizes the effector protein Avr-blb2 which is secreted by *P. infestans* (Bozkurt et al., 2011). The effector recognized by the AMR3 protein is Avr-amr3 (Lin et al., 2022). This recognition leads to the activation of the hypersensitive response (Oh et al., 2009, 2014). The hypersensitive response is a form of programmed cell death characterized by cytoplasmic shrinkage, chromatin condensation, mitochondrial swelling, vacuolization and chloroplast disruption (Coll et al., 2011). Although BLB2 and AMR3 recognition is specific for Avr-blb2 and Avr-amr3, respectively, the hypersensitive response pathway in plants is conserved (Baker et al., 1997; Feys and Parker, 2000). This pathway involves signaling proteins specific to plants; activated R-proteins cannot trigger cell death in mammals (Coll et al., 2011). Thus, BLB2 and AMR3 do not have a toxic mechanism of action, but instead activate signaling cascades within the plant which invoke the plant cell death pathway to prevent growth and spread of the pathogen.

Protein expression levels

An attempt was made to detect BLB2 and AMR3 in leaf and tuber tissue of the transformed potato line BG25 using Western detection method and specific antibodies to short peptides located in the C-terminal portion of these proteins (MRIDs 52055401 and 52055402). The limits of quantification were established at 250 parts ppb FW in leaf and 500 ppb FW in tuber tissues for AMR3, and at 250 ppb FW in leaf and 100 ppb FW in tuber tissues for BLB2. Both the AMR3 and BLB2 protein expression levels were below the limits of detection. R-proteins are known to have extremely low levels of expression and kept under tight control to prevent spurious activation of cell death pathways in plant tissues (Marone et al., 2013). These low levels of expression of BLB2 and AMR3 are thus an intrinsic quality of R-proteins.

Thus, this data supports very low human exposure to BLB2 and AMR3 through the consumption of BLB2 and AMR3 expressing potatoes.

Bioinformatic searches for similarity to known toxins

The protein sequences of BLB2 and AMR3 were analyzed for similarity to known toxins by performing searches against a toxin database (MRIDs 51073805, 51529201, 52055404 and 52086801). The toxin database was generated by filtering the UniProtKB database using a text-based, keyword search for “toxin”, and contained 807,537 sequences. The toxin homology searches were performed using BLAST (blastp; https://blast.ncbi.nlm.nih.gov/Blast.cgi) with an E-value cutoff of 10^{-1} (MRID 51529201) and 10^{-2} (MRIDs 51073805, 52055404 and 52086801). Based on an analysis of the output, the Agency concludes that BLB2 and AMR3 do not demonstrate significant homology to known toxins.

History of safe use

The BLB2 protein is encoded by the *Rpi-blb2* gene which originates from *S. bulbocastanum*. The protein sequence is 82% identical to the tomato gene Mi-1 (van der Vossen et al., 2005), which has a history of safe use as tomatoes have been consumed by humans for hundreds of years. The BLB2 protein is present in two *S. tuberosum* potato varieties (Toluca and Bionica) cultivated for food use in Europe (Haverkort, 2009). These varieties provide a history of safe use of BLB2-expressing potatoes.

AMR3 is encoded by *Rpi-amr3* gene which originates from *Solanum americanum* (Witek, 2016). *S. americanum* is found naturally growing on disturbed ground, but is also cultivated for medicinal and food use (Särkinen et al., 2018), and as part of breeding programs for improved nutrition (Dinssa et al., 2016). The leaves and berries of *S. americanum* are eaten throughout Africa and Asia (Edmonds and Chweya, 1997) but consumption patterns do not include tubers (which *S. americanum* does not produce). Since leaves are a primary target for infection by *Phythophthora infestans*, they are likely to contain higher levels of AMR3 relative to other tissue although, given generally very low levels of expression of R-proteins in plants to prevent activation of programmed cell death in the absence of stimuli (Marone et al., 2013), it is likely that AMR3 levels are below detectable levels in all tissues. In the US, there are anecdotal reports of *S. americanum* being both poisonous (https://plants.ces.ncsu.edu/plants/solanum-americanum/), and edible (https://www.eattheweeds.com/american-nightshade-a-much-maligned-edible/). To explain these discrepancies, it is possible that the toxicity of the plant varies with the stage of development, geographical location, and, in the case of human cultivation, with human-imposed selection (Särkinen et al., 2018). However, the toxicity of the *Solanum* genus is well described to be caused by glycoalkaloids (Milner et al., 2011), which can cause toxicity even in the common *Solanum tuberosum* (Korpan et al., 2004). AMR3 is not a glycoalkaloid and instead belongs to a large family of NBS-LRR R-proteins found throughout the plant kingdom; there are hundreds to thousands of R-proteins in *S. tuberosum* and other crops which have a long history of safe consumption (Meyers et al., 1999).

2. Allergenicity profile

Allergenicity was examined using a "weight-of-evidence" approach based on the source of the trait and the amino acid sequence compared with known allergens. Based on the analysis below, EPA concluded that the potential for BLB2 or AMR3 to be a food allergen is minimal.

Source organism

As discussed in the section *History of safe use* above, the source of BLB2 is *S. bulbocastanum* (van der Vossen et al., 2005). The BLB2 protein is present in two *S. tuberosum* potato varieties (Toluca and Bionica) cultivated for food use in Europe (Haverkort, 2009). Although uncommon, there exist allergies to *S. tuberosum*, which are caused by protein patatin and, possibly, other compounds (Tomás-Pérez et al., 2019). Other than the allergy to *S. tuberosum* in general, no allergy reports can be found for the two varieties known to contain the BLB2 protein when the MEDLINE database (https://pubmed.ncbi.nlm.nih.gov/) was searched using terms "Toluca" or "Bionica", and "allergy".

AMR3 originates from *S. americanum* (Witek, 2016). No allergy cases reports can be found for *S. americanum* when MEDLINE database (https://pubmed.ncbi.nlm.nih.gov/) was searched using terms "*Solanum americanum*" and "allergy".

Bioinformatic searches for similarity to known allergens

Searches were performed to identify homology between BLB2 or AMR3 and known allergens in the AllergenOnline.org database (MRIDs 51073805 and 51529201) and COMPARE database (MRIDs 52055404 and 52086801). Two searches were performed: one using the 80-mer sliding window search and one using an 8-mer exact match search. The 80-mer sliding window search identifies localized regions of similarity by comparing contiguous 80 amino acid sequences from each protein to sequences in the AllergenOnline or COMPARE databases. Matches were defined as sequences having greater than 35% identity to known allergens. The 8-mer exact-match search identifies small, localized regions consisting of eight amino acids of identity between the queried sequence and known or suspected allergens in the databases. The length of the identity stretch for this search is based on the expected size of IgE epitopes. While IgE epitopes have been reported to be as short as five amino acids (Banerjee et al., 1999; Beezhold et al., 1999), more often characterized IgE linear epitopes are eight amino acids or longer (Chatchatee et al., 2001; Shin et al., 1998).

No known allergens were identified that matched the amino acid sequences of the BLB2 or AMR3 proteins using the 80-mer or 8-mer searches in the AllergenOnline or COMPARE databases above the set thresholds.

B. PVY Coat Protein hairpin RNA

The PVY Coat Protein hairpin RNA is from a sequence based on the Potato virus Y (PVY) viral coat protein gene and is designed to target the coat protein region of the PVY RNA genome. The introduced genetic cassette in the Gen 3 potato uses the natural defense pathway in plants to protect the plant prior to viral infection. The genetic cassette transcribes a dsRNA hairpin from a sequence based on the PVY viral coat protein. The PVY coat protein DNA is oriented as an inverted repeat in the Gen 3 potato which results in the production of dsRNA but not production of a protein. The transcribed dsRNA is processed within the plant's RNAi pathway into siRNAs. The siRNAs are then already present in the plant before a PVY infection occurs so that the cells are primed to actively protect the plant as soon as PVY infects. As the siRNAs produced from the dsRNA hairpin are sequence-specific to the PVY coat protein, they then target the viral RNA genome for degradation and block viral replication, thus conferring PVY protection.

The 2005 FIFRA Scientific Advisory Panel (SAP) confirmed that plant viral coat proteins (PVCP) would not be anticipated to present hazards, stating that virus coat proteins ''are naturally present in the environment and no adverse effects to humans or non-targets have been reported'' (FIFRA SAP meeting held December 6-7, 2005, page 12 of minutes). Although the aforementioned statement is specific to viral coat proteins rather than dsRNA, the 2005 SAP also opined on post transcriptional gene silencing (PTGS) mechanisms targeting plant viruses, of which the PVY coat protein DNA inverted repeat and transcribed dsRNA hairpin uses. The 2005 SAP stated that, "PTGS is a highly desirable strategy for generating virus resistant plants for several reasons. First, this strategy is based on a natural plant defense mechanism against viruses. Second, transgenes can easily be designed to produce only RNA, not protein. Third, PTGS gives

stronger resistance than protein-based methods. Because of all these advantages, this is the premier virus resistance strategy for use today and into the future." (FIFRA SAP 2005, page 40 of minutes). In terms of risk of a PTGS mechanism for viral protection, the 2005 SAP also recommended, "exempting [from regulations under FIFRA] any PIP designed to induce PTGS that does not encode a viral protein" (FIFRA SAP 2005, page 12 of minutes).

Although the components of siRNA-producing machinery exist both in plants and in mammals, both long hairpin RNA and plant-derived siRNAs are unlikely to be functional in mammalian (and human) cells. Mammalian Dicer, the enzyme that cleaves hairpin RNAs for production of siRNAs, is inefficient in processing long hairpin RNAs (Ma et al., 2008). In addition, IFN system in mammalian cells, which is the preferred anti-viral defense in mammals, actively inhibits processing of hairpin RNA into siRNAs (Van Der Veen et al., 2018). siRNAs produced from plant hairpin transgenes and extracted from plants are also ineffective for gene silencing in mammalian cells due to several structural differences (Chau and Lee, 2007).

Finally, several physiological and biochemical barriers may play a role in preventing the uptake of plant RNAs by mammalian cells. Nucleases in the saliva break down the food-derived miRNAs after ingestion (Rodrigues & Petrick, 2020). As ingested food reaches the stomach, degradation of dietary miRNAs occurs due to the low pH and hydrolysis by digestive enzymes (Huang et al., 2018; Rodrigues & Petrick, 2020). Delivery of plant RNA via the oral route is difficult to achieve due to rapid degradation and poor transcytosis across the mammalian gut (Petrick et al., 2013).

Therefore, due to the differences between vertebrates and plants in generation and structure of siRNAs, and vertebrate physiological barriers preventing uptake of orally ingested RNAs, coupled with the long history of exposure of humans and vertebrate animals to in planta RNA, there is the reasonable expectation that PVY Coat Protein hairpin RNA in the Gen 3 potato poses no hazard to humans.

C. StmALS

1. Toxicological profile

Mammalian toxicity was examined via a "weight-of-evidence" approach using mode of action, expression levels, history of safe use, and bioinformatics. Based on the analysis below, StmALS protein represents a negligible risk to human or livestock that consume potato products.

Mode of action

StmALS confers herbicide tolerance in the Gen 3 potato. StmALS is proposed for use as an inert ingredient and does not have any pesticidal activity of its own. StmALS differs from the native potato ALS (StALS) by two amino acid substitutions: the tryptophan residue at 563 changed to leucine, and the serine residue at 642 changed to isoleucine (W563L, S642I). ALS catalyzes the first common step in the synthesis of the branched chain amino acids, isoleucine, leucine, and valine. ALS converts two pyruvate molecules to 2-acetolactate (a precursor of leucine and valine), or pyruvate and 2-ketobutyrate to 2-aceto-2-hydroxy-butyrate.

Sulfonylureas and imidazolinone herbicides inhibit branched chain amino acid synthesis by binding to ALS and blocking substrate (i.e., pyruvate or 2-ketobutyrate) access to the active site (McCourt et al., 2006). By blocking the first step in branched chain amino acid synthesis, these ALS inhibitors cause a deficiency in amino acids necessary for growth and survival, and an accumulation of 2-ketobutyrate (Duggleby and Pang, 2000), which results in the death of the plant. Herbicide tolerance conferred by StmALS is due to amino acid substitutions that interfere with herbicide binding. This allows the substrates to enter the active site even in the presence of the herbicide, ensuring the synthesis of branched chain amino acids.

Thus, the herbicide tolerance mode of action of StmALS is not toxic.

Protein expression levels

The StmALS protein was detected by Western blot using a specific antibody and quantified using densitometry in the transformed potato BG25 leaf and tuber samples collected from four different field locations. The average StmALS protein expression level was 1,830 ppb FW in leaf (range 780-6,010 ppb FW) and 420 ppb FW in tubers (range 200-570 ppb FW) (MRID 51976802).

Bioinformatic searches for similarity to known toxins

Searches for homology of StmALS protein to known toxins were performed against a toxin database generated by filtering the UniProtKB database using a text-based, keyword search for "toxin." The toxin database contains 807,537 sequences that were downloaded as a FASTA file and used to build a BLAST database using the makeblastdb.exe that is part of Blast 2.9.0+. The toxin homology searches were performed using BLAST (blastp; https://blast.ncbi.nlm.nih.gov/Blast.cgi) with an E-value cutoffs of 10^{-1} (MRID 51529202) or 10^{-2} (MRIDs 51073808, 52055404 and 52086801). Based on an analysis of the output, the Agency concludes that StmALS does not demonstrate significant homology to known toxins.

History of safe use

StmALS is 99.7% identical to the native potato ALS, which has a long history of safe use. Two mutations have been introduced into the StmALS sequence: the tryptophan residue at 563 changed to leucine, and the serine residue at 642 changed to isoleucine (W563L, S642I).

The history of safe use of the native potato StALS applies to the StmALS toxicity, since two amino acid substitutions which do not affect StALS mode of action are not likely to result in the production of a toxic protein.

2. *Allergenicity profile*

Allergenicity was examined using a "weight-of-evidence" approach based on the source of the trait, the amino acid sequence compared with known allergens, and the biochemical properties of the protein, including in vitro digestibility in simulated gastric fluid (SGF), heat stability, and glycosylation. Based on the analysis below, EPA concluded that the potential for StmALS to be a food allergen is minimal.

Source organism

StmALS is derived from the *S. tuberosum* ALS (StALS) and differs from it by two amino acid substitutions: the tryptophan residue at 563 changed to leucine, and the serine residue at 642 changed to isoleucine (W563L, S642I). These substitutions render StmALS tolerant to sulfonylureas and imidazolinone herbicides, which inhibit StALS.

Although it is not likely that the two amino acid substitutions would result in the production of a toxic protein as they do not affect StALS mode of action, the difference in two amino acids in StmALS could conceivably imbue the modified enzyme with different allergenic properties; for this reason, the applicant was asked to provide the data of all the below tests which together constitute the weight-of-evidence approach to allergenicity.

Glycosylation

Current scientific knowledge suggests that protein glycosylation may contribute to protein stability and enhance its allergenic potential (Pedrosa et al., 2000; Wormald et al., 1999; Shreffler et al., 2006). For this reason, the Agency uses glycosylation status as part of the weight-of-evidence approach in evaluation of the allergenic potential of a protein.

No glycosylation could be detected for StmALS protein using SDS-PAGE with appropriate controls (MRID 51785602).

The conclusion that StmALS is not glycosylated is strengthened by two additional considerations. First, no N-X-T/S sites of N-glycosylation, or the expanded consensus sequence for N-glycosylation sites N-X-T/S/C (Medzihradszky, 2008), were identified in StmALS protein sequence. Secondly, the glycosylation status of ALS from soybean, which is similar (~85% amino acid identity) to the potato derived StALS, was previously evaluated by EPA and found to not be glycosylated (USEPA, 2012).

The lack of experimentally detected glycosylation of StmALS, in conjunction with the protein sequence analysis and previously demonstrated absence of glycosylation of a related protein in soybean, indicate that StmALS is not glycosylated.

Equivalency of the bacterially-expressed and plant-expressed StmALS protein used in stability studies

The recombinant StmALS purified from *E. coli* was used in heat stability and stability to digestion by gastric proteases assays. The recombinant StmALS was expressed in *E. coli* without the chloroplast transit peptide, but as the chloroplast transit peptide is also expected to be naturally cleaved off StmALS, analysis of the thermostability and stability to gastric proteases of StmALS without the chloroplast transit peptide is acceptable.

The equivalency of the recombinant StmALS to the plant-expressed StmALS was confirmed by the recombinant StmALS being active in the colorimetric assay that relies on conversion of acetolactate to acetoin by the enzymatic activity of StmALS (MRID 52086800). This

observation confirms that recombinant StmALS possesses enzymatic properties comparable to those of the plant-produced StmALS.

Heat stability

Heat treatments such as baking, cooking, roasting, pasteurization and others reduce allergen stability and activity by chemical modification, unfolding protein structure and/or aggregation with the food matrix (Besler et al., 2001). For this reason, stability of proteins to heat treatment included in the weight-of-evidence arguments in assessment of their allergenicity (Codex Alimentarius, 2003).

The heat lability of recombinant StmALS was assessed by two corroborative methods (MRID 52086800).

The first method relies on detection of protein band after exposure to heat with protein aggregation and precipitation acting as a proxy for denaturation. Denaturation of the potential allergen would result in the loss of conformational epitopes that could be targets of recognition by IgE in allergenic response (Pekar et al., 2018). StmALS band on Western blot clearly disappears after 1hr incubation at 90 ºC, due to denaturation, aggregation and precipitation.

The second method measured enzymatic activity after heating to test StmALS denaturation by heat treatment. Complete loss of StmALS activity was observed after heating for 20 minutes at temperatures above 56 °C. Attenuation of StmALS activity with increasing temperature is indicative of protein unfolding and denaturation.

These data demonstrate that the soluble form of StmALS protein is denatured and becomes insoluble after heat treatment. Since potatoes are cooked by frying, boiling, or baking at high temperatures, and not consumed raw, StmALS is expected to become denatured during potato processing.

Stability to the digestion by gastric proteases

One of the main characteristics of proteins triggering an allergic response via the gastrointestinal tract is resistance to gastrointestinal digestion (Pekar et al., 2018). Stability of proteins to digestion by gastric proteases is, therefore, part of the weight-of-evidence arguments in assessment of their allergenicity.

The stability of recombinant StmALS to digestion by gastric proteases such as pepsin and pancreatin was assayed in MRID 52086800. Pepsin is a protease produced by the stomach, whereas pancreatin is a mixture of several digestive enzymes produced by the exocrine cells of the pancreas, including proteases trypsin and chymotrypsin, peptidase, lipase, amylase and bile salts. The pancreas secretes these enzymes into duodenum, immediately past the stomach. Proteases present in the duodenum further break down dietary proteins into small peptides or amino acids to facilitate intestinal absorption (Wang et al., 2020).

Full-length StmALS was hydrolyzed in less than 30 seconds by either pepsin or pancreatin. During digestion with pepsin, a transient fragment of about 15 kDa derived from StmALS was

observed at 0.5 and 2 minutes of digestion, however, it disappeared at the 5-minute timepoint and was not observed later. There were no products which can be attributed to partial digestion of StmALS observed during pancreatin digestion of StmALS.

Rapid and complete digestion of the StmALS protein by either stomach or pancreatic proteases suggests that StmALS or its fragments would not persist in the small intestine sufficiently long to interact with the immune system and induce allergy.

Bioinformatic searches for similarity to known allergens

Searches were performed to identify homology between StmALS and known allergens in the AllergenOnline.org database (MRIDs 51073808 and 51529202) and COMPARE database (MRIDs 52055404 and 52086801). Two searches were performed: one using the 80-mer sliding window search and one using an 8-mer exact match search. The 80-mer sliding window search identifies localized regions of similarity by comparing contiguous 80 amino acid sequences from each protein to sequences in the AllergenOnline or COMPARE databases. Matches were defined as sequences having greater than 35% identity to known allergens. The 8-mer exact-match search identifies small, localized regions consisting of eight amino acids of identity between the queried sequence and known or suspected allergens in the databases. The length of the identity stretch for this search is based on the expected size of IgE epitopes. While IgE epitopes have been reported to be as short as five amino acids (Banerjee et al., 1999; Beezhold et al., 1999), more often characterized IgE linear epitopes are eight amino acids or longer (Chatchatee et al., 2001; Shin et al., 1998).

No known allergens were identified that matched the amino acid sequence of the StmALS protein using the 80-mer or 8-mer searches in the AllergenOnline or COMPARE databases above the set thresholds.

V. Human Exposure and Risk Characterization Assessment

A. Federal Food, Drug, and Cosmetic Act (FFDCA) Considerations

1. Toxicological Profile and Allergenicity Assessment Conclusions

BLB2 and AMR3

The Agency used a "weight-of-evidence" approach consistent with the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants" to conclude that AMR3 and BLB2 do not exhibit toxic or allergenic potential. The information presented in support of this registration application is adequate to inform the human health risk assessment for the temporary tolerance exemption under the standards the Food Quality Protection Act (FQPA). Based on the reviewed information, BLB2 and AMR3 proteins represent a negligible risk to human or livestock that consume potato products.

VNT1

An exemption from the requirement of a tolerance has previously been granted for the *Rpi-vnt1* gene that expresses the VNT1 protein (40 CFR §174.534). EPA's analysis confirms that this established exemption is applicable to the *Rpi-vnt1* gene and VNT1 protein expressed in Gen 3 potatoes.

PVY Coat Protein hairpin RNA

EPA has established an exemption from the requirement of a tolerance for residues of nucleic acids that are part of a plant-incorporated protectant (40 CFR § 174.507). EPA's analysis confirms that this established exemption is applicable to the PVY Coat Protein hairpin RNA expressed in Gen 3 potatoes.

StmALS

The Agency used a "weight-of-evidence" approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants" and considered evidence from data on history of safe use, source organism, mode of action, glycosylation assay, stability to heat and gastric proteases assay and bioinformatic searches to conclude that StmALS does not exhibit toxic or allergenic potential. The information presented in support of this registration application is adequate to inform the human health risk assessment for the permanent tolerance exemption under the standards the Food Quality Protection Act (FQPA). Based on the reviewed information, StmALS protein represents a negligible risk to human or livestock that consume potato products.

2. Aggregate Exposure and Risk Characterization, Not Including Occupational Exposure

BLB2, AMR3, and StmALS

In examining aggregate exposure, EPA considers available information concerning exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectant chemical residue, and exposure from non-occupational sources.

No adverse effects of concern for BLB2, AMR3 and StmALS were expected based on history of safe use, origin of traits, and bioinformatics search for homology with known toxins and allergens; therefore, the EPA did not conduct a quantitative exposure assessment.

a. Food Exposure

BLB2 and AMR3

BLB2 and AMR3 can be consumed with potato tuber-derived food, such as cooked potatoes (fries, chips, hash browns etc), dry flakes, and flour present in infant food. To a lesser extent, food exposure may occur with consumption of potato starch (obtained from potato tubers), due to some contamination with non-starch (protein) ingredients. Since the levels of BLB2 and AMR3 in potato tubers were found to be below the LOQ (100 ppb FW for BLB2 and 500 ppb FW for AMR3, MRIDs 52055401 and 52055402), the exposure is expected to be minimal.

Based on the history of safe use and bioinformatics analysis of the traits, the EPA has concluded that BLB2 and AMR3 are not toxic or allergenic to humans. The hazard to human health from food exposure to BLB2 and AMR3 is minimal. Due to the minimal hazard to human health from BLB2 and AMR3, and to the minimal exposure, the risk from food exposure to these PIPs is expected to be minimal.

StmALS

StmALS can be consumed with potato tuber-derived food, such as cooked potatoes (fries, chips, hash browns etc), dry flakes, and flour present in infant food. To a lesser extent, food exposure may occur with consumption of potato starch (obtained from potato tubers), due to some contamination with non-starch (protein) ingredients. In all cases, potatoes are subjected to a heat treatment before consumption. StmALS has been shown to be heat labile, which would reduce any toxicity or allergenicity potential, if present, and mitigate the exposure. Food consumption of StmALS is expected to amount to an exposure level of 0.0061-0.031 mg/kg bw/d.

Based on the description of origins of StmALS, history of safe use, absence of homology to known toxins and allergens, lack of glycosylation and susceptibility to the digestion by gastric proteases and heat, the EPA has concluded that StmALS is not toxic or allergenic to humans. The hazard to human health from food exposure to StmALS is minimal. Due to the minimal hazard to human health from StmALS, the risk from food exposure is expected to be minimal.

b. Drinking Water Exposure

BLB2 and AMR3

Oral exposure from ingestion of drinking water is unlikely because BLB2 and AMR3 are present at very low levels within the plant cells. If AMR3 and BLB2 do enter the water column, they are expected to degrade rapidly in the presence of soil microbes, or upon normal communal water-treatment procedures. AMR3 and BLB2 are not likely to contaminate the sources of drinking water or pass the typical drinking water treatment.

StmALS

Oral exposure from ingestion of drinking water is unlikely because StmALS is present at low levels and confined within the plant cells. If StmALS does enter the water column, it is expected to degrade rapidly in the presence of soil microbes, or upon normal communal water-treatment

procedures. StmALS is not likely to contaminate the sources of drinking water or pass the typical drinking water treatment.

c. Non-Occupational and Residential Exposure

BLB2 and AMR3

Non-occupational or residential exposure, other than dietary, is expected to be minimal given that the active ingredients are a plant-incorporated protectant in potato. Therefore, the only possible route of non-occupational exposure, other than dietary, is via handling of the plants and plant products. Exposure via inhalation is not likely since BLB2 and AMR3 are contained within plant cells, which essentially eliminates this exposure route or reduces it to negligible levels. Non-dietary exposure via the skin is somewhat more likely via the contact with potato products which might have been processed in a way that disrupts cellular structure; however BLB2 and AMR3 are present in the transformed potato tissues at levels below LOD, resulting in minimal to negligible exposure. Furthermore, there are no risks associated with these exposure routes because based on bioinformatics analysis and history of use, the proteins are not toxic or allergenic.

StmALS

Non-occupational or residential exposure, other than dietary, is expected to be minimal given that the inert is a plant-incorporated protectant in potato. Therefore, the only possible route of non-occupational exposure, other than dietary, is via handling of the plants and plant products. Exposure via inhalation is not likely since StmALS contained within plant cells, which essentially eliminates this exposure route or reduces it to negligible levels. Non-dietary exposure via the skin is somewhat more likely via the contact with potato products which might have been processed in a way that disrupts cellular structure. The most likely way plant proteins in general can have an effect via dermal exposure is by eliciting an allergic reaction (Barata and Conde-Salazar, 2013); however, bioinformatic analysis, history of use, and biochemical and stability assays indicate that StmALS is not allergenic. EPA therefore concludes that there will be no risks associated with dermal exposure to StmALS.

2. Cumulative Effects

BLB2, AMR3, and StmALS

Section 408(b)(2)(D)(v) of FFDCA requires that, when considering whether to establish, modify, or revoke a tolerance, EPA consider "available information concerning the cumulative effects of residues and other substances that have a common mechanism of toxicity." Based EPA analyses, there is no indication of mammalian toxicity resulting from the plant-incorporated protectants or inert. In the absence of such effects, EPA concludes that there are no identifiable cumulative effects for the BLB2, AMR3, or StmALS proteins.

3. Determination of Safety for U.S. Population, Infants and Children

a. U.S. Population

BLB2, AMR3, and StmALS

For all of the reasons discussed previously, EPA concludes that there is reasonable certainty that no harm will result to the U.S. population, including infants and children, from aggregate exposure to residues of BLB2, AMR3, and StmALS. This includes all anticipated dietary exposures and all other exposures for which there is reliable information.

b. Infants and Children

BLB2, AMR3, and StmALS

For all of the reasons discussed previously, EPA has concluded that BLB2, AMR3, and StmALS are not toxic or allergenic to mammals, including infants and children. Because there are no threshold levels of concern to infants, children, and adults, EPA concludes that no additional margin of safety is necessary to protect infants and children.

B. Occupational Exposure and Risk Characterization

BLB2, AMR3, and PVY Coat Protein hairpin RNA

Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible levels. Potatoes are not propagated via seed, so worker's exposure via seed dust is not an issue. Therefore, the Agency concludes that there will be minimal occupational exposure. If exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity of BLB2, AMR3, and the PVY Coat Protein hairpin RNA.

StmALS

Exposure via the skin or inhalation is not likely since StmALS is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible levels. Potatoes are not propagated via seed, so worker's exposure via seed dust is not an issue. Therefore, the Agency concludes that there will be minimal occupational exposure. If exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity of the StmALS protein.

C. Residue Analytical Methods

BLB2 and AMR3

RT-qPCR method was developed for detection of *Rpi-amr3* and *Rpi-blb2* transcripts. The method adequately detected *Rpi-amr3* and *Rpi-blb2* mRNAs, which is supported by detection of

Rpi-amr3 and *Rpi-blb2* mRNAs in BG25 leaves and tubers, but not in the leaves and tubers of the untransformed Russet Burbank potato.

The petitioner is committed to provide reference material, appropriate control substances and technical support in the future in case of potential recall.

StmALS

Immunoblot assay was developed for detection of StmALS with an antibody that was specific to potato StmALS and did not show cross reactivity to native potato ALS. The assay adequately detects StmALS, which is supported by the fact that pSIM4363-transformed event BG25 contained detectable levels of StmALS in leaf and tuber tissues, whereas StmALS was not detected in the untransformed potato leaf and tuber tissues.

The petitioner is committed to provide reference material, appropriate control substances and technical support in the future in case of potential recall.

D. Human Health Risk Assessment Conclusion

BLB2 and AMR3

Based on the molecular characterization data, expression data, human health rationale, and bioinformatics analysis for BLB2 protein and AMR3 protein expressed in the Gen 3 potato, EPA has determined that no unreasonable adverse effects to humans are expected from BLB2 and AMR3 proteins and the genetic material necessary for their production in the Gen 3 potato during the proposed EUP. Based on the reviewed information, BLB2 and AMR3 proteins represent a negligible risk to human or livestock that consume potato products.

PVY Coat Protein hairpin RNA

Based on the molecular characterization data, expression data and human health rationale for the PVY Coat Protein hairpin RNA expressed in the Gen 3 potato, EPA has determined that no unreasonable adverse effects to humans are expected from the PVY Coat Protein hairpin and the genetic material necessary for its production in the Gen 3 potato during the proposed EUP. Further, the existing tolerance exemption for residues of nucleic acids that are part of a plant-incorporated protectant (40 CFR § 174.507) is applicable to the PVY Coat Protein hairpin expressed in the Gen 3 potato, indicating a negligible risk to human or livestock that consume potato products.

StmALS

Based on the molecular characterization data, expression data, human health rationale, bioinformatics analysis, and biochemical and stability data for the StmALS protein in the Gen 3 potato, EPA has determined that no unreasonable adverse effects to humans are expected from the StmALS protein and the genetic material necessary for its production in the Gen 3 potato during the proposed EUP. Based on the reviewed information, the StmALS protein represents a negligible risk to human or livestock that consume potato products.

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