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

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

# MEMORANDUM

**SUBJECT:** Human Health Risk Assessment and Review of Product Characterization of the Insecticidal Plant-Incorporated Protectants, *Pseudomonas chlororaphis* IPD072Aa protein and DvSSJ1 dsRNA Complementary to the *DvSSJ1* Gene Sequence from *Diabrotica virgifera virgifera,* and the Genetic Material Necessary (vector PHP74643) for their Production in Event DP23211 Maize (OECD Unique ID DP-Ø23211-2) and Establishment of a Permanent Tolerance Exemption. Data were provided in support of a FIFRA Section 3 Seed Increase Registration.

| | |
|---|---|
| **File Symbol:** | **29964-ET** |
| **Submission No.:** | **1037533** |
| **Petition No.:** | **9F8785** |
| **Parent Case** | **00016641** |
| **Action Code Case** | **00135361** |
| **PC Codes:** | **006569 (IPD072Aa) and 006568 (DvSSJ1)** |
| **MRID Nos.:** | **50844701-03, 50844745-50, 50844752-62, 50844777-88, 51014202-07, 51014209, 51521506, 51645602, 51804804, 52194201-03** |

**FROM:** Nina Ortiz, Ph.D., Biologist
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

NINA ORTIZ Digitally signed by NINA ORTIZ Date: 2024.03.25 15:31:09 -04'00'

**THRU:** Amanda Pierce, Ph.D., Senior Scientist
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

AMANDA PIERCE Digitally signed by AMANDA PIERCE Date: 2024.03.25 15:39:41 -04'00'

Michael Mendelsohn, Chief
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division

MICHAEL MENDELSOHN Digitally signed by MICHAEL MENDELSOHN Date: 2024.03.26 16:49:26 -04'00'

**TO:** Matthew Weiner, Risk Manager
Emerging Technologies Branch
Biopesticides and Pollution Prevention Division (7511P)

## I. Executive Summary

Pioneer Hi-Bred International, Inc. (Pioneer, or the applicant) submitted information in support of a FIFRA Section 3 seed increase registration for the plant-incorporated protectant designated as event DP-Ø23211-2 in maize (referred to as DP23211 maize). DP23211 maize expresses the new plant-incorporated protectant (PIP) active ingredients IPD072Aa protein and DvSSJ1 double-stranded RNA (dsRNA) as well as the PIP inert ingredients phosphinothricin acetyltransferase protein (PAT) and the phosphomannose isomerase (PMI) protein (Table 1 below). The gene for the insecticidal protein IPD072Aa was derived from *Pseudomonas chlororaphis* and is intended to provide protection from certain coleopteran pests through disruption of the midgut epithelium. The DvSSJ1 dsRNA transcript targets *Diabrotica virgifera virgifera* (Western Corn Rootworm, WCR) and functions by weakening smooth septate junctions in the insect gut via RNA interference (RNAi). The PIP inert ingredients found in DP23211 maize, PAT and PMI, were used as selectable markers. The PAT protein confers tolerance to glufosinate herbicides and the PMI protein allows the plant to metabolize mannose as a carbon source.

Pioneer Hi-Bred International has submitted a petition for the establishment of a tolerance exemption for the IPD072Aa protein in or on the food and feed commodities of corn: corn, field; corn, sweet; and corn, pop. Per 40 CFR §174.507, EPA established a tolerance exemption for residues of nucleic acids that are part of a PIP which would apply to the DvSSJ1 dsRNA found in DP23211 maize. Additionally, the PIP inert ingredients, PAT and PMI, found in DP23211 maize meet the criteria for exemption from the requirement of a tolerance per 40 CFR § 174.522 (PAT) and 40 CFR § 174.527 (PMI) (USEPA 2023).

**TABLE 1. Plant-Incorporated Protectants in DP23211 Maize and Their Target Pests.**

| COMMON NAME | PROTEIN | PC CODE | TARGET PEST |
|---|---|---|---|
| IPD072Aa | IPD072Aa protein | 006569 | Active ingredient - Targets Coleopterans by disrupting midgut epithelium |
| DvSSJ1 | DvSSJ1 dsRNA | 006568 | Active ingredient -Targets *Diabrotica virgifera virgifera* by weakening insect gut septate junctions |
| PAT | phosphinothricin acetyltransferase protein | 817305 | Inert ingredient - selectable marker- tolerance to glufosinate herbicide |
| PMI | phosphomannose isomerase protein | 706505 | Inert ingredient - selectable marker- confers ability to metabolize mannose as carbon source |

The molecular characterization and human health data submitted to support the FIFRA Section 3 seed increase registration request for the new plant-incorporated protectant, IPD072Aa protein and the DvSSJ1 dsRNA, in DP23211 maize has been determined as acceptable and thus sufficient to support the applicant's registration request. This finding was made based on the following information:

IPD072Aa Protein

- Acute oral toxicity studies in mice classified IPD072Aa protein as EPA Toxicity Category III
- Bioinformatics studies indicated no similarity to known toxins or allergens
- Demonstrated lack of glycosylation
- Complete digestion in both simulated gastric fluid containing pepsin and simulated intestinal fluid containing pancreatin
- Corn products are typically not consumed raw and often prepared at temperatures of 100 °C or higher and heat stability studies indicated IPD072Aa protein loses its stability at temperatures over 95 °C

DvSSJ1 dsRNA

- Mode of action specific to Western corn rootworm
- Presence of physiological barriers in humans and other vertebrates mitigate uptake of plant RNA in mammalian cells
- Existing tolerance exemption for residues of nucleic acids that are part of a plant-incorporated protectant (40 CFR § 174.507)

The 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 IPD072Aa protein and the genetic material necessary for its production in maize event DP23211. This includes all the anticipated dietary exposure and all other exposures for which there is reliable information. Therefore, the data submitted for maize event DP23211 are acceptable to support the petition for an exemption from the requirement of a tolerance for residues of IPD072Aa protein in or on the food and feed commodities of corn under the standards the Food Quality Protection Act (FQPA).

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

**DATA REVIEW RECORD**

## II. Product Characterization

### A. Background

Pioneer Hi-Bred International, Inc. (Pioneer, the applicant, or the registrant) is seeking a FIFRA Section 3 seed increase registration for the PIP designated as event DP-Ø23211-2 in maize (referred to as DP23211 maize) and a petition for a permanent exemption from tolerance for the residues of IPD072Aa protein in or on the food and feed commodities of corn. DP23211 maize expresses the new plant-incorporated protectant (PIP) active ingredients IPD072Aa protein and the DvSSJ1 double-stranded RNA (dsRNA) as well as the PIP inert ingredients phosphinothricin acetyltransferase protein (PAT) and the phosphomannose isomerase (PMI) protein.

The IPD072Aa protein produced in DP23211 maize is an insecticidal protein whose gene was originally isolated from *Pseudomonas chlororaphis* (Schellenberger *et al.,* 2016). *P. chlororaphis* is a ubiquitous plant and soil bacterium that is more commonly found in the root environment of several plants and is well known for its biocontrol abilities (Arrebola *et al*., 2019; Chin-A-Woeng *et al*., 2001). The first product using *P. chlororaphis* as an active ingredient was registered with the EPA in September 2001 (EPA Reg. No.: 75801-2) and this bacterium has not demonstrated toxicity or pathogenicity to humans, wildlife, or the environment. Under field conditions, the IPD072Aa protein protects against feeding damage from certain coleopterans by way of midgut epithelium disruption.

The DvSSJ1 dsRNA produced in DP23211 maize is intended to target and suppress the mRNA transcript for the *dvssj1* gene in *Diabrotica virgifera virgifera* (or Western Corn Rootworm; WCR), a gene encoding a membrane protein that is specific to arthropods and integral in cell-to-cell junctions that are required for arthropod intestinal barrier function (Hu *et al*., 2016; Furuse *et al*., 2017). The DvSSJ1 dsRNA in DP23211 maize suppresses the target mRNA transcript in WCR through RNA interference (RNAi), a mechanism which regulates gene expression and defense against transposable elements and RNA-based viruses in almost all eukaryotic organisms (Torri *et al*., 2022). Suppression of the *dvssj1* transcript through RNAi results in injury to the WCR midgut epithelium that is WCR-specific.

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). The *dvssj1* gene and DvSSJ1 dsRNA are both composed of nucleic acids and covered by the tolerance exemption at 40 CFR 174.507. Therefore, no petition to establish a tolerance exemption was necessary, and EPA has not conducted an assessment under FFDCA for DvSSJ1 dsRNA as expressed in DP23211 maize.

### B. The Transformation System

DP23211 maize was created by site-specific integration (SSI) using two sequential transformation steps. The first transformation step utilized microprojectile bombardment to insert an integration site sequence (referred to as a “landing pad” sequence) at a specific location of the

maize genome. The landing pad then serves as the location for inserting the desired genetic elements in a subsequent transformation. The second step utilized *Agrobacterium*-mediated transformation with a plasmid containing the target T-DNA in addition to several genetic elements. This transformation step inserted the intended expression cassettes into the landing pad in the maize genome. Integration of the T-DNA region from the plasmid into the landing pad occurred via flippase-mediated recombination. After each transformation step, the maize genome was characterized using Southern-by-Sequencing (SbS™) to ensure the intended insertion was present and there were no unintended plasmid-derived sequences present in the genome.

## C. Characterization of the DNA Inserted in the Plant

Southern-by-Sequencing analysis (SbS™ technology; SbS) was conducted on the T1 generation of DP23211 maize to determine the insertion copy number and intactness as well as confirm the absence of any plasmid backbone. This was done by collecting genomic DNA from the leaf tissue of ten DP23211 maize samples and one control maize plant. Using full coverage probes containing the sequences of all plasmids used in the transformation of DP23211, SbS analysis indicated that the T1 generation of DP23211 maize contains a single, intact copy of the intended insertion and that no additional insertions or plasmid backbone sequences are present in the DP23211 genome.

An analysis of the genomic flanking regions was conducted to assess whether any fusion proteins were created via novel open reading frames as a result of the DNA inserted in the DP23211 maize genome. To this end, the 5' and the 3' flanking genomic border sequences through the DP23211 insertion sequence (covering a total of 500 base pairs on each border) of DP23211 maize were subjected to BLAST analysis. Two novel open reading frames (ORFs) were identified- one at the 5' genomic border to insert region and the second at the 3' genomic border to insert region, spanning 43 and 16 amino acids in length, respectively. However, no promotors were identified upstream of the ORFs and it is unlikely that transcription would occur and thus the production of a fusion protein in DP23211 is not expected.

Five generations of DP23211 maize were examined for Mendelian inheritance of event DP-Ø23211-2 via genotypic and phenotypic analyses. The genotypic analysis used quantitative polymerase chain reaction (qPCR) to evaluate for the consistent placement in the genome and presence of the insertion site (*E10360.104.1.12*) as well as evaluate the presence of the genes intended for insertion (*ipd072*, *mo-pat*, *pmi*, *DvSSJ1)*. The phenotypic analysis evaluated tolerance to glufosinate and support Mendelian inheritance of event DP23211 maize. Further, SbS analysis was conducted on five generations of DP23211 maize and demonstrated that the inserted DNA remained stable across five subsequent generations. Figure 1 below illustrates a schematic map of the intended DP23211 insertion.

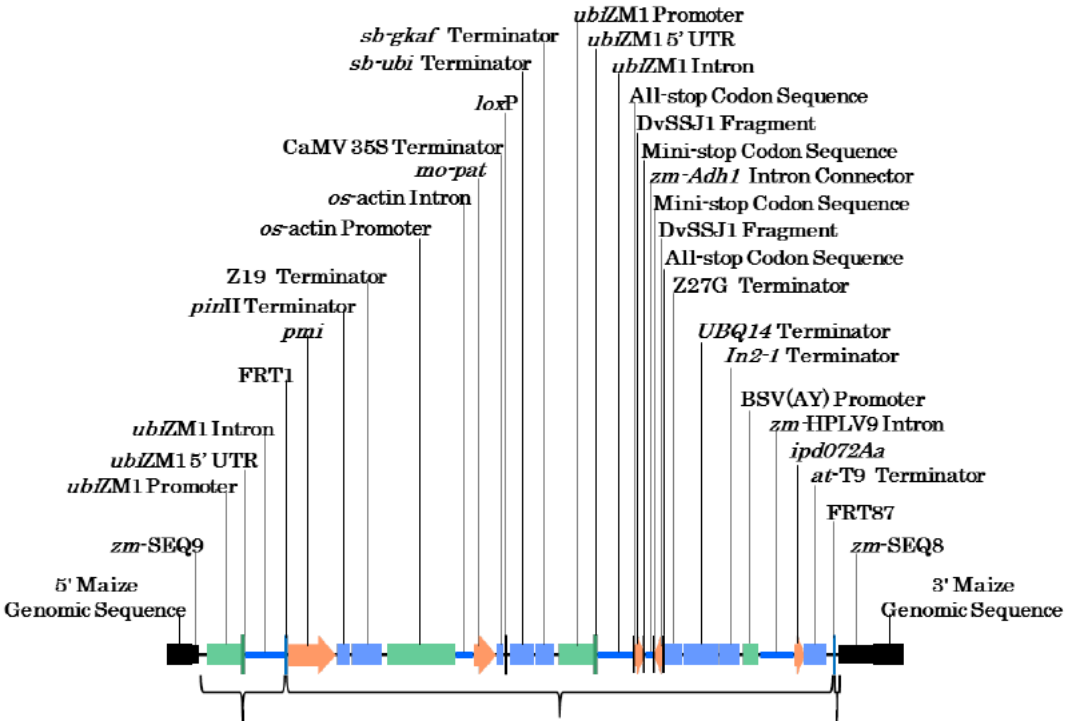


**Figure 1. Map of the Intended DP23211 Insertion**
Schematic map of the insertion intended to be present (bracketed regions) in the DP23211 maize genome. The size of the intended insertion is 16,176 bp. The flanking maize genomic regions are represented by horizontal black bars.

## D. Protein Characterization and Expression

A study was conducted to assess the expression profile of IPD072Aa and DvSSJ1 dsRNA in various DP23211 plant tissue samples. Conducted during the 2018 growing season at six sites in the United States and one site in Canada, tissue samples were collected from DP23211 maize, DP23211 maize treated with glufosinate (referred to as herbicide-treated DP23211 maize), and non-genetically modified (non-GM) near-isoline control maize (referred to as control maize). The tissue samples included those from root (V6, V9, R1, R4, and R6 growth stages), leaf (V9, R1, R4, and R6 growth stages), pollen (R1 growth stage), forage (R4 growth stage), whole plant (R1 and R6 growth stages), and grain (R6 growth stage). Protein concentrations for IPD072Aa protein, PAT protein, and PMI protein were measured using quantitative enzyme-linked immunosorbent assay (ELISA). For the DvSSJ1 dsRNA, the QuantiGene Plex Assay was used to determine dsRNA concentration.

The concentration of IPD072Aa protein varied among different tissue types in non-herbicide treated samples, with the highest mean concentration found in root (42 ng/mg-dw; R6) and the lowest mean concentration found in pollen (0.76 ng/mg-dw; R1). Grain had a mean concentration of 2.6 ng/mg-dw (R6), the highest mean concentration found in leaf was 18 ng/mg-dw (R1), the highest mean concentration found in whole plant was 15 ng/mg-dw (R6), and forage had a mean concentration of 22 ng/mg-dw (R4). Protein levels in non-herbicide treated samples were similar to those found in the herbicide treated samples, by tissue type. Protein concentration values calculated for IPD072Aa were corrected for extraction efficiency.

DvSSJ1 dsRNA concentration in DP23211 maize was relatively similar across tissue types with the highest mean concentration measured in leaf ($6.46 \times 10^{-2}$ µg/g-dw; R4) and the lowest mean concentration found in pollen ($9.87 \times 10^{-4}$ µg/g-dw; R1). Grain had a mean concentration of $4.13 \times 10^{-3}$ µg/g-dw (R6), the highest mean concentration found in root was $5.13\ 10^{-2}$ µg/g-dw (V6), the mean concentration found in forage was $1.90 \times 10^{-2}$ µg/g-dw (R4), and the highest concentration found in whole plant was $2.19 \times 10^{-2}$ µg/g-dw (R1). DvSSJ1 dsRNA levels in non-herbicide treated samples were similar to those found in the herbicide treated samples. In addition, the quantities of DvSSJ1 dsRNA were similar for dry and fresh tissues. DvSSJ1 dsRNA levels determined in this study were not corrected for extraction efficiency as was done for determining protein concentration of IPD072Aa.

Characterization studies were conducted on the microbially-expressed IPD072Aa and its bioactivity was also assessed. Bioactivity analysis was conducted for IPD072Aa to determine if microbially expressed IPD072Aa protein would be a suitable surrogate for DP23211-maize derived IPD072Aa. In this study, *D. virgifera virgifera* larvae were fed IPD072Aa protein derived from either DP23211 maize or purified from a microbial expression system, at three different concentrations: 12.5 µg/cm$^2$, 25 µg/cm$^2$, and 50 µg/cm$^2$. Survival and weight of the larvae were assessed after 7 days. Based on the observations of these studies, the microbially-produced IPD072Aa protein was demonstrated to be a suitable surrogate for the DP23211-maize derived protein, with overlapping Clopper-Pearson 95% confidence intervals and comparable mortality observed between microbially-produced lots and across the microbially-produced and maize-derived IPD072Aa protein samples. These studies indicated that the microbially-expressed IPD072Aa protein were found to be suitable surrogates for DP23211-expressed IPD072Aa, as the characterization studies found that the microbially-expressed and DP23211-isolated IPD072Aa proteins were biochemically and functionally identical. It should be noted that the microbially-expressed IPD072Aa retained an N-terminal histidine tag after purification, an amino acid that is not present in the N-terminal sequence of DP23211 maize-isolated IPD072Aa protein. However, the characterization and bioactivity studies conducted using the microbially-expressed surrogate protein support the finding that the presence of the N-terminal histidine tag does not affect the functionality of IPD072Aa.

Bioactivity was also assessed for the DvSSJ1 dsRNA found in DP23211 maize. Similar to the bioactivity study for IPD072Aa, this study orally exposed *D. virgifera virgifera* larvae to DvSSJ1 dsRNA to three *in vitro*-synthesized DvSSJ1 dsRNA test substances and one DP23211 maize-derived DvSSJ1 dsRNA test substance. Five concentrations were evaluated for each test substance: 1.7, 3.3, 6.6, 13.2, and 26.5 femtomole (fmol). The $EC_{50}$ values for all four test substances had similar level of weight inhibition and overlapping $EC_{50}$ confidence intervals, suggesting all three *in vitro*-synthesized DvSSJ1 dsRNA test substances represent conservative surrogates with respect to bioactivity for the DP23211 maize-derived DvSSJ1 dsRNA.

## E. Supporting Data

The product characterization studies submitted to support the registrant's application for the FIFRA Section 3 seed increase registration for the new PIP active ingredients IPD072Aa protein and DvSSJ1 dsRNA, as expressed in DP23211 maize, and associated FFDCA petition to establish a permanent exemption from the requirement of a tolerance for residues of IPD072Aa

protein in food and feed commodities of corn are summarized with their classifications in Table 2. 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 FIFRA section 3 seed increase registration of the new PIPs, IPD072Aa and DvSSJ1 dsRNA and the genetic material necessary for their production in DP23211 maize, and the associated FFDCA petition for a permanent tolerance exemption for IPD072Aa.

**Table 2. Summary of Product Identity, Manufacturing Process, and Analytical Methods Data Submitted in Support of the Section 3 Registration of DP23211 Maize Containing IPD072Aa Protein and DvSSJ1 dsRNA.**

| STUDY TYPE | RESULT | MRID NO. |
|---|---|---|
| Segregation Analysis and Tissue Production of Multiple Maize Generations Containing Event DP-Ø23211-2 | Genotypic and phenotypic analyses were conducted for five generations of DP23211 maize: BC1F1 (in genetic background PH1V5T), BC2F1, T1, T5, and BC1F1 (in genetic background PH2SRH). Genotypic analysis utilized quantitative polymerase chain reaction (qPCR) and endpoint PCR to evaluate each individual plant for the presence of the insertion site E10360.104.1.12 and genes intended for insertion into DP23211 maize: *ipd072, mo-pat, pmi,* and *DvSSJ1*. Phenotypic analysis evaluated tolerance to glufosinate for each individual plant. Genotypic and phenotypic results generated in this study demonstrated Mendelian inheritance of event DP23211 maize in three segregating generations (BC1F1, BC2F1, and T1) and one non-segregating generation (T5). For each individual plant in all generations, the genotypic result was the same as the corresponding phenotypic result, indicating the DNA insertion co-segregated with the trait phenotype and was stable through traditional breeding.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844701 |
| Description and Sequence of the T-DNA Region from Plasmid PHP74643 | This study provided detailed information on the genetic elements present in the plasmid used to transform maize (*Zea mays* L.) via *Agrobacterium*-mediated transformation. The T-DNA of this plasmid expresses several gene cassettes as well as the RNA fragment cassette.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844702 |
| Characterization of IPD072Aa Protein Derived from a Microbial Expression System | These studies characterized the IPD072Aa protein derived from a microbial expression system for two different lots of proteins (PCF-0037-AP and PCF-0040) as described in MRIDs 50844746 and 50844749, respectively. Characterization of the IPD072Aa protein was achieved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, western blot analysis, protein glycosylation analysis, amino acid composition analysis, mass determination of the intact protein by mass spectrometry, mass determination of chymotryptic peptides by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS/MS), N-terminal amino acid sequencing, and bioactivity assay methods. The microbially derived IPD072Aa protein had the expected molecular weight, immunoreactivity, amino acid sequence, bioactivity, and lack of glycosylation. The endotoxin content of the IPD072Aa protein preparation was also determined and the preparation demonstrated acceptable endotoxin content. This microbially-expressed protein did retain an N-terminal histidine tag post purification. | 50844746<br>50844749 |

**Table 2. Summary of Product Identity, Manufacturing Process, and Analytical Methods Data Submitted in Support of the Section 3 Registration of DP23211 Maize Containing IPD072Aa Protein and DvSSJ1 dsRNA.**

| STUDY TYPE | RESULT | MRID NO. |
|---|---|---|
| | **CLASSIFICATION: ACCEPTABLE** | |
| Molecular Characterization and Biochemical Equivalency of DvSSJ1 dsRNA | The DvSSJ1 fragment cassette within DP23211 maize is expressed as a 901-nucleotide (901-nt) transcript. The functional region of the 901-nt DvSSJ1 transcript consists of 210-basepairs (210-bp) of dsRNA arranged in an inverted repeat configuration. The DvSSJ1_210 dsRNA region is designed to target a 210-bp region of the smooth septate junction protein 1 (*dvssj1*) gene in western corn rootworm (WCR, *Diabrotica virgifera virgifera*).<br><br>The studies conducted in these MRIDs assessed the concentration and purity of the *in vitro* transcribed DvSSJ1_210 base pair double stranded RNA (dsRNA) test material, Lot# FGN-10.07.2016 and Lot# FGN-01.25.2018. Additionally, these studies verified the sequence of the DP23211-derived DvSSJ1 dsRNA and *in vitro* transcribed DvSSJ1 dsRNA test samples which include both the 210-bp sequences and 901-bp samples. *In vitro* samples were found to be adequately characterized and thus suitable for use in future studies.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844750<br>50844755<br>50844761<br>50844786 |
| Expressed Trait Protein and RNA Concentrations of a Maize Line Containing Event DP-Ø23211-2 | This study measured the protein concentrations of IPD072Aa, PAT, and PMI and the DvSSJ1 dsRNA concentration expressed in tissues derived from DP23211 maize. The following samples were collected from DP23211 maize, DP23211 maize treated with glufosinate, and non-genetically modified (non-GM) near-isoline control maize (i.e., control maize): root (V6, V9, R1, R4, and R6 growth stages), leaf (V9, R1, R4, and R6 growth stages), pollen (R1 growth stage), forage (R4 growth stage), whole plant (R1 and R6 growth stages) and grain (R6 growth stage). Samples were analyzed for IPD072Aa, PAT, and PMI protein concentrations using quantitative enzyme-linked immunosorbent assay (ELISA) methods and analyzed for DvSSJ1 dsRNA concentration using the QuantiGene Plex Assay. Results were provided as means, ranges, and standard deviations.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844754 |
| Characterization of PMI Protein Derived from DP-Ø23211-2 Maize | The objective of this study was to partially purify and then characterize the PMI protein expressed in DP23211 maize. The PMI protein was partially purified from DP23211 maize whole plant tissue using ammonium sulfate precipitation and immuno-affinity chromatography. Characterization was achieved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, western blot analysis, protein glycosylation analysis, and peptide mapping by liquid chromatography-mass spectrometry (LC-MS). The PMI protein derived from DP23211 maize had the expected molecular weight, immunoreactivity, amino acid sequence, and lack of glycosylation.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844756 |
| Characterization of PAT Protein Derived from DP-Ø23211-2 Maize | The objective of this study was to partially purify and then characterize the PAT protein expressed in DP23211 maize. The PAT protein was partially purified from DP23211 maize whole plant tissue using ammonium sulfate precipitation, immuno-affinity chromatography, and | 50844757 |

**Table 2. Summary of Product Identity, Manufacturing Process, and Analytical Methods Data Submitted in Support of the Section 3 Registration of DP23211 Maize Containing IPD072Aa Protein and DvSSJ1 dsRNA.**

| STUDY TYPE | RESULT | MRID NO. |
|---|---|---|
| | ion exchange chromatography. Characterization was achieved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, western blot analysis, protein glycosylation analysis, peptide mapping by liquid chromatography-mass spectrometry (LC-MS), and N-terminal amino acid sequencing. The PAT protein derived from DP23211 maize had the expected molecular weight, immunoreactivity, amino acid sequence, and lack of glycosylation.<br><br>**CLASSIFICATION: ACCEPTABLE** | |
| Characterization of IPD072Aa Protein Derived from DP-Ø23211-2 Maize | The objective of this study was to partially purify and then characterize the IPD072Aa protein expressed in DP23211 maize. The IPD072Aa protein was partially purified from DP23211 maize whole plant tissue using ammonium sulfate precipitation and immuno-affinity chromatography. Characterization was achieved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, western blot analysis, protein glycosylation analysis, peptide mapping by liquid chromatography-mass spectrometry (LC-MS), and N-terminal amino acid sequencing. The IPD072Aa protein derived from DP23211 maize had the expected molecular weight, immunoreactivity, amino acid sequence, and lack of glycosylation.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844758 |
| Characterization of DP-023211-2 Maize for Insertion Stability in Five Generations Using Southern Blot Analysis | Southern blot analysis was conducted on five generations of DP23211 maize to demonstrate the inserted DNA remained stable across multiple subsequent generations. Genomic DNA samples from individual plants of the Tl, T2, T3, T4, and T5 generations of DP23211 maize and control maize were analyzed by digestion with restriction enzyme *Kpn* I and hybridization with the *pmi, mo-pat* and *ipd072Aa* gene and DvSSJl fragment probes. The presence of equivalent bands from hybridization with the *pmi, mo-pat*, *ipd072Aa* and DvSSJ1 fragment probes within all five generations analyzed confirms that the inserted DNA in DP23211 maize is stable and equivalent across multiple generations during the breeding process.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844760 |
| Description of Transformation Method and Familiarity of PAT and PMI Proteins for Maize Event DP-Ø23211-2 | This MRID provided a description of the transformation method used to develop DP23211 maize, expressing IPD072Aa protein, DvSSJ1 double-stranded RNA (dsRNA), PAT protein for tolerance to glufosinate herbicide, and PMI protein used as a selectable marker. Transformation occurred as two sequential transformation steps to (1) insert a specific integration site sequence in the maize genome (referred to as a "landing pad"), and (2) insert the intended trait genes into the landing pad using site-specific integration (SSI). Following each transformation, verification of the intended insertion was confirmed via Southern-by-Sequencing (SbS™). SbS analyses can be found in MRID 50844759.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844785 |
| Validation of QuantiGene Plex Assay for | The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the | 50844787 |

**Table 2. Summary of Product Identity, Manufacturing Process, and Analytical Methods Data Submitted in Support of the Section 3 Registration of DP23211 Maize Containing IPD072Aa Protein and DvSSJ1 dsRNA.**

| STUDY TYPE | RESULT | MRID NO. |
|---|---|---|
| Quantification of DvSS1 Double-stranded RNA from Maize Tissues | measurement of multiple RNA transcripts simultaneously. It should be noted that although the assay allows for multiplexing, only detection of the 901 nt transcript of DvSSJ1 RNA was validated in this assay. The DvSSJ1 QuantiGene assay was developed, optimized, and validated to quantify the concentration of DvSSJ1 double-stranded RNA (dsRNA) in total RNA isolated from the following maize tissues expressing DvSSJ1: forage, whole plant, grain, pollen, root, leaf. The results of the validation demonstrate that the methods described are suitable for the quantification of DvSSJ1 dsRNA from maize tissues.<br><br>**CLASSIFICATION: ACCEPTABLE** | |
| Southern-by-Sequencing Analysis of the T1 Generation of DP-Ø23211-2 Maize | This report contains a detailed description of the characterization of DP23211 maize to determine the copy number of the insert and to demonstrate the absence of unintended plasmid sequences in the DP23211 genome. Additionally, the 5' and 3' genomic border sequences were subject to BLAST analyses to determine the location of the intended insertion into the maize genome and a flanking region analysis was conducted to assess for the possibility of novel open reading frames (ORFs) as a result of the DNA being inserted into the genome. Results demonstrated that DP23211 maize contains a single, intact copy of the intended insertion, no additional insertions or plasmid backbone sequences are present in its genome, and functional ORFs capable of producing novel proteins were not identified.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844759<br>52194201<br>52194202 |
| Enzyme-Linked Immunosorbent Assay (ELISA) Method Validation Summary for IPD072Aa, PAT, and PMI Proteins in Lyophilized Maize Tissues | Enzyme-linked immunosorbent assays (ELISAs) are commonly used to quantify the expression levels of proteins in plant tissues. Pioneer developed an ELISA method, which incorporates the reagents from an IPD072Aa protein ELISA kit produced by EnviroLogix Inc. (Portland, ME, USA), to specifically quantify the concentration of IPD072Aa protein in lyophilized maize tissues. Additionally, Pioneer developed an ELISA method to specifically quantify the concentration of PAT and PMI proteins in lyophilized maize tissues. These reports summarize the results of the internal validation experiments conducted by Pioneer to verify the method performs as intended. The assays were validated using representative maize leaf, pollen, root, whole plant, and grain samples. Method validation included an evaluation of assay matrix effects, specificity, accuracy, repeatability (precision), dilution agreement, and extraction efficiency. Results from this validation demonstrated that the ELISA methods are suitable for quantification of IPD072Aa, PAT, and PMI proteins in lyophilized maize tissues.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844777<br>50844778<br>50844779 |
| Lateral Flow Test Kit Method for the Detection of IPD072Aa Protein in Maize Grain | Lateral flow test kits are commonly used to determine the presence or absence of plant-incorporated protectants (PIPs) proteins. DP23211 maize expresses the IPD072Aa protein to provide control of corn rootworms. Pioneer has developed a lateral flow test kit for the detection of IPD072Aa protein in maize grain samples and is in the process of having the kit GIPSA certified. This report summarizes their methodology to be used for the analysis of maize grain. | 51014209 |

**Table 2. Summary of Product Identity, Manufacturing Process, and Analytical Methods Data Submitted in Support of the Section 3 Registration of DP23211 Maize Containing IPD072Aa Protein and DvSSJ1 dsRNA.**

| STUDY TYPE | RESULT | MRID NO. |
|---|---|---|
| | **CLASSIFICATION: ACCEPTABLE** | |
| Bioactivity Analysis of IPD072Aa Protein Lots PCF-0037-AP, PCF-0040, and PRCH-4044 Derived from a Microbial Expression System | This study evaluated the bioactivity of IPD072Aa protein lots PRCH-4044, PCF-0037-AP, and PCF-0040 derived from a microbial expression system. Diets containing three different concentrations from each lot (12.5 µg/cm$^2$, 25 µg/cm$^2$, and 50 µg/cm$^2$) were fed to *Diabrotica virgifera virgifera* larvae (*D. virgifera virgifera*) and survival and weight were assessed after 7 days. *D. virgifera virgifera* mortality was comparable for each of the three IPD072Aa protein lots (PRCH-4044, PCF-0037-AP, and PCF-0040) at each target overlay protein concentration. The 95% confidence levels for mortality overlap at each of the three IPD072Aa protein concentrations for each protein lot. Therefore, all three microbially derived IPD072Aa test substances represent conservative surrogates with respect to bioactivity for the DP23211 maize-derived IPD072Aa.<br><br>**CLASSIFICATION: ACCEPTABLE** | 51521506<br>51645602 |
| Evaluation of Bioactivity of Several DvSSJ1 double-stranded RNA Test Substances | This study used *Diabrotica virgifera virgifera* larvae to evaluate bioactivity of three *in vitro*-synthesized DvSSJ1 dsRNA test substances and one DP23211 maize-derived DvSSJ1 dsRNA test substance by assessing survival and weight after 14 days. Five concentrations were evaluated for each test substance: 1.7, 3.3, 6.6, 13.2, and 26.5 femtomole (fmol). For the control group, the control diet, RNase-free water was used. The reference control diet was total RNA isolated from non-genetically-modified (non-GM) maize tissue. Because of the variability between lots, the $LC_{50}$ was found to be an unreliable indicator of biological effect due to the test substance for this particular study. Therefore, the $EC_{50}$ was assessed and weight inhibition was demonstrated in all treatments with the test substances. The $EC_{50}$ values for all four test substances had similar level of weight inhibition and overlapping $EC_{50}$ confidence intervals, suggesting that all three *in vitro*-synthesized DvSSJ1 dsRNA test substances represent conservative surrogates with respect to bioactivity for the DP23211 maize-derived DvSSJ1 dsRNA. Because the $LC_{50}$ did not conclusively indicate *Diabrotica virgifera virgifera* mortality as a result of exposure to the test substances, this study is classified as supplemental.<br><br>**CLASSIFICATION: SUPPLEMENTAL** | 51804804<br>52194201 |

## III. Hazard Analysis

Following the requirements as specified in 40 CFR § 158.500, mammalian toxicity studies were submitted to support the application for the FIFRA Section 3 seed increase registration for the PIP based on the active ingredients IPD072Aa protein and DvSSJ1 dsRNA as expressed in DP23211 maize and associated FFDCA petition to establish a permanent exemption from the requirement of a tolerance for residues of the IPD072Aa protein in food and feed commodities of corn. These studies are summarized with their classifications in Table 3, below. For the IPD072Aa protein. scientific rationales were submitted in support of the Acute Dermal Toxicity (OCSPP 870.1200), Acute Inhalation Toxicity (OCSPP 870.1300), and Acute Eye Irritation

(OCSPP 870.2400). For the DvSSJ1 dsRNA, rationales were submitted for Acute Oral Toxicity (OCSPP 870.1100), Acute Dermal Toxicity (OCSPP 870.1200), Acute Inhalation Toxicity (OCSPP 870.1300), and Acute Eye Irritation (OCSPP 870.2400). Mammalian toxicity studies were submitted in support of the oral exposure pathway for IPD072Aa protein (OCSPP 870.1100). 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 3 seed increase registration of the PIPs and FFDCA petition for a permanent exemption from the requirement of a tolerance for IPD072Aa. Further testing at higher toxicological tiers is not required.

**Table 3. Summary of Mammalian Toxicity Data Submitted in Support of the Section 3 Registration of PIPs IPD072Aa Protein and DvSSJ1 dsRNA in DP23211 Maize.**

| STUDY TYPE | RESULT | MRID NO. |
|---|---|---|
| IPD072Aa Protein: Acute Oral Toxicity Study in Mice | In this study, groups of fasted, 7-week-old Crl:CD1(ICR) mice (6 males and 6 females) were exposed to IPD072Aa protein by oral gavage at a concentration of 2000 mg/kg bw and observed for 15 days. Under the conditions of this study, intragastric exposure of IPD072Aa protein to male and female mice at a single dose of 2000 mg/kg did not result in mortality or other evidence of acute oral toxicity, and the oral $LD_{50}$ of the test substance is >2000 mg/kg bw for both sexes of mice tested.<br><br>**EPA Toxicity Category III**<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844745 |
| IPD072Aa Protein Waiver Request for Acute Dermal Toxicity, Acute Inhalation Toxicity, and Acute Eye Irritation | The IPD072Aa protein is derived from a ubiquitous bacterium (*Pseudomonas chlororaphis*) that has a history of use in agriculture with a narrow spectrum of activity. This information, in combination with submitted studies demonstrating lack of effects observed in laboratory testing and the lack of direct exposure via the dermal, pulmonary, and ocular routes, suggests that acute toxicity to humans and animals is not expected. Thus, the rationales included in these MRIDs are adequate to support waiving the requirements for acute dermal, acute inhalation, and acute eye irritation testing with IPD072Aa protein, as they would not be additive to the overall safety assessment.<br><br>**CLASSIFICATION: ACCEPTABLE** | 51014202<br>51014203<br>51014204 |
| Determination of the Biological Activity of Heat-Treated IPD072Aa Protein Incorporated in an Artificial Diet and Fed to *Diabrotica virgifera virgifera* | This study evaluated the biological activity of heat-treated IPD072Aa protein when incorporated in an artificial diet fed to *D. virgifera virgifera*. Larvae were exposed via oral ingestion to one of seven heat-treated IPD072Aa samples (control diet, unheated, 25 °C, 50 °C, 60 °C, 95 °C, 121 °C). Results demonstrated that IPD072Aa protein autoclaved for approximately 30 minutes at 121 °C became inactive against *D. virgifera virgifera* when incorporated in an artificial diet. Some decrease in activity for IPD072Aa protein heat-treated for approximately 30 minutes at 25-95 °C (Treatments 3-6) was | 50844703 |

**Table 3. Summary of Mammalian Toxicity Data Submitted in Support of the Section 3 Registration of PIPs IPD072Aa Protein and DvSSJ1 dsRNA in DP23211 Maize.**

| STUDY TYPE | RESULT | MRID NO. |
|---|---|---|
| | observed although the difference in activity between Treatments 3-6 and Treatment 2 (unheated) was not statistically significant.<br><br>**CLASSIFICATION: ACCEPTABLE** | |
| Characterization of the *In Vitro* Pepsin and Pancreatin Resistance of IPD072Aa Using SDS-PAGE and Western Blot Analysis | *In vitro* pepsin and pancreatin resistance of IPD072Aa protein to simulated gastric (SGF) fluid and simulated intestinal fluid (SIF) was followed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. IPD072Aa was incubated for 0, 0.5, 1, 2, 5, 10, 20, 30, and 60 minutes in SGF containing pepsin at pH ~1.2 and SIF containing pancreatin at pH 7.5. For the pepsin analysis, the IPD072Aa protein was rapidly digested (within 0.5 minutes) in SGF. For the pancreatin analysis, the IPD072Aa protein was digested in SIF in 20 minutes.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844747<br>50844748 |
| Comparison of the IPD072Aa Protein Sequence to the Protein Sequences in the DuPont Pioneer Toxin Database | Potential toxicity of the IPD072Aa protein was assessed by comparison of its sequence to the sequences in the DuPont Pioneer toxin database, a database produced by filtering the proteins in the UniProtKB/Swiss-Prot database for molecular function by keywords that could imply toxicity or adverse health effects (e.g., toxin, hemagglutinin, vasoactive, etc.). The search between the IPD072Aa protein sequence and protein sequences in the database was conducted with BLASTP using default parameters, except that low complexity filtering was turned off, the *E*-value threshold was set to $10^{-4}$, and unlimited alignments were returned.<br><br>No alignments with an *E*-value $\leq 10^{-4}$ were returned between the IPD072Aa protein sequence and any protein sequence in the DuPont Pioneer toxin database. Therefore, no toxicity concerns arose from the bioinformatics assessment of the IPD072Aa protein.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844752 |
| Comparison of the Amino Acid Sequence of the IPD072Aa Protein to the Amino Acid Sequences of Known and Putative Protein Allergens | Two separate searches for the IPD072Aa protein sequence were performed using the Comprehensive Protein Allergen Resource (COMPARE) 2023 database (January 26, 2023). The first search examined the IPD072Aa protein sequence against the allergen sequences in the database to identify any alignments that are a length of 80 amino acids or greater and possess a sequence identity of >35%. The second search was used to identify any contiguous 8-residue identical matches between the IPD072Aa protein sequence and the allergen sequences. The results of this study found no alignments that were a length of 80 or greater with a sequence identity of ≥ 35% nor were contiguous 8-residue matches between the IPD072Aa protein sequence and the allergen sequences identified. Taken together, the comparisons of the IPD072Aa protein sequence to the allergen sequences showed that there is no apparent allergenicity concern *in silico* regarding the IPD072Aa protein.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844753 |

| Table 3. Summary of Mammalian Toxicity Data Submitted in Support of the Section 3 Registration of PIPs IPD072Aa Protein and DvSSJ1 dsRNA in DP23211 Maize. | | |
|---|---|---|
| **STUDY TYPE** | **RESULT** | **MRID NO.** |
| Comparison of the DvSSJ1 Fragment to the Human Transcriptome | The 210 nt single-stranded DvSSJ1 sequence (both sense and anti-sense) were compared to the sequences in the human transcriptome to identify any 21-nucleotide (21-nt) exact matches which could indicate potential off-target effects in humans. No perfect 21-nt matches were identified between either the sense or anti-sense DvSSJ1 fragments and any transcript in the human transcriptome. This bioinformatic study indicates that the potential for off-target effects of the DvSSJ1 fragment in humans *in silico* is negligible. However, a more thorough analysis to assess for potential off-target effects *in silico* would allow for 0-2 nucleotide mismatches between the DvSSJ1 transcripts and the human transcriptome. For this reason, this study was classified as supplemental.<br><br>**CLASSIFICATION: SUPPLEMENTAL** | 50844762 |
| DvSSJ1 dsRNA Waiver Request for Acute Oral Toxicity, Acute Dermal Toxicity, Acute Inhalation Toxicity, and Acute Eye Irritation | The MRIDs reviewed in this DER provided rationales in support of waiving the study requirements for DvSSJ1 dsRNA expressed in DP23211 maize for the following studies: Acute Oral Toxicity, Acute Dermal Toxicity, Acute Inhalation Toxicity, and Acute Eye Irritation (OCSPP Guidelines: 870.1100, 870.1200, 870.1300, 870.2400, respectively). The rationales relied on the history of safe consumption of dsRNA in human and animal diets, the specificity of the activity of DvSSJ1 dsRNA, which is limited to *Diabrotica* within the order Coleoptera, the low estimated exposure of DvSSJ1 dsRNA to humans and livestock due to multiple physical, enzymatic, biochemical, and molecular barriers to exposure, and the bioinformatic analysis which revealed the absence of exact nucleotide matches between DvSSJ1 dsRNA and the human transcriptome. The information presented in these MRIDs suggest that toxicity of DvSSJ1 dsRNA to humans and animals via the oral, dermal, pulmonary, and ocular routes of exposure is not expected and these acute toxicity studies would not be additive to the overall safety assessment. Thus, the waivers for these requirements are justified.<br><br>**CLASSIFICATION: ACCEPTABLE** | 50844788<br>51014205<br>51014206<br>51014207 |

## A. IPD072Aa Protein

### 1. Toxicological Profile

Mammalian toxicity was examined via a "weight of evidence" approach using acute oral toxicity, mode of action, and bioinformatics of IPD072Aa. Based on the analysis below, the IPD072Aa protein represents a negligible risk to humans or livestock that consume DP23211 maize.

*Acute oral toxicity*
A synergism study was evaluated in the associated environmental risk assessment to assess the combined potency of IPD072Aa protein and DvSSJ1 dsRNA in DP23211 maize using western

corn rootworm, an insect sensitive to the two active ingredients (USEPA 2024). A greater than additive effect when used in combination on the target pest was observed. Because IPD072Aa functions within the pest midgut where it breaks down the midgut epithelial lining, it is likely that the activity of the pesticidal protein results in higher rates of uptake of the dsRNA in the gut of the target pest, thus resulting in greater target pest mortality when used in combination than either active ingredient in isolation. However, because DvSSJ1 is highly specific to the *Diabrotica* genus on a genetic level and because there is no pathway for the activity spectrum of DvSSJ1 to expand due to synergy with IPD072Aa, there is no expectation that the combined use of these active ingredients in DP23211 corn would alter the hazard characterization compared to the active ingredients in isolation.

Therefore, an acute oral toxicity study in mice was performed with IPD072Aa protein in isolation using microbially-expressed IPD072Aa protein. The results of this study found that intragastric exposure of IPD072Aa protein to male and female mice at a single dose of 2000 mg/kg did not result in mortality or other evidence of acute oral toxicity. The oral $LD_{50}$ was determined to be >2000 mg/kg bw for both sexes of mice tested, and therefore is classified as EPA Toxicity Category III in mice. As mentioned above, this study used microbially-expressed IPD072Aa, as utilizing DP23211-derived IPD072Aa protein for this study was not feasible since sufficient amounts of protein could not be extracted from DP23211 maize.

*Mode of action*
The insecticidal protein, IPD072Aa, was derived from *Pseudomonas chlororaphis* and confers protection from certain coleopteran pests by causing disruption of the midgut epithelium. The IPD072Aa protein has a narrow spectrum of activity which is specific to the target organism (formally discussed in the ecological risk assessment associated with this application; USEPA 2024), further lowering the likelihood that IPD072Aa would pose a hazard to humans or livestock that consume DP23211 maize.

*Bioinformatic searches for similarity to known toxins*
The potential toxicity of IPD072Aa was examined *in silico* using the internal DuPont Pioneer toxin database, based on the proteins found in the UniProtKB/Swiss-Prot database. This search was conducted with BLASTP using default parameters and an *E*-value threshold set to $10^{-4}$. There were no alignments returned which met these criteria, suggesting that the IPD072Aa protein does not raise toxicity concerns, *in silico*.

## 2. Allergenicity Profile

Allergenicity was examined using a "weight-of-evidence" approach based on the source of the trait, the amino acid sequence of the protein compared with known allergens, and the biochemical properties of the protein, including *in vitro* digestibility in simulated gastric and intestinal fluids (SGF and SIF), glycosylation, and heat stability. Based on the analysis below, EPA concluded that the potential for IPD072Aa to be a food allergen is minimal.

*Source of the trait*
The IPD072Aa protein produced in DP23211 maize is an insecticidal protein whose gene was originally isolated from *Pseudomonas chlororaphis* (Schellenberger et al., 2016). *P.*

*chlororaphis* is a ubiquitous plant and soil bacterium that is more commonly found in the root environment of several plants and is well known for its biocontrol abilities (Arrebola et al., 2019; Chin-A-Woeng et al., 2001). The first product using *P. chlororaphis* as an active ingredient was registered with the EPA in September 2001 (EPA Reg. No.: 75801-2) and this bacterium has not demonstrated toxicity or pathogenicity to humans, wildlife, or the environment. Therefore, *Pseudomonas chlororaphis* is not considered to be a source of allergenic proteins.

*Amino acid sequence*
The potential to induce allergenicity for IPD072Aa protein was assessed *in silico* via bioinformatics. The Comprehensive Protein Allergen Resource (COMPARE) 2023 database (updated January 26, 2023; available at http://comparedatabase.org) was used to identify any alignments sliding over a length of 80 amino acids or greater that possess a sequence identity of >35%. Additionally, a second search was used to identify any contiguous 8-residue identical matches between the IPD072Aa protein sequence and the allergen sequences. This study found no alignments meeting either criteria, suggesting it does not represent an allergenicity concern *in silico*.

*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.

*In vitro* digestibility studies using the microbially-expressed IPD072Aa protein found that simulated gastric fluid (SGF) containing the enzyme pepsin digested the protein within 0.5 minutes and simulated intestinal fluid (SIF) containing the enzyme pancreatin digested the protein within 20 minutes. Complete degradation of IPD072Aa in the simulated gastric environment indicated that the intact protein will not pass from the stomach into the intestinal lumen and therefore indicates a low possibility to induce an allergic response via the gastrointestinal tract.

*Glycosylation*
Current scientific knowledge suggests that protein glycosylation may contribute to protein stability and enhance its allergenic potential. Therefore, the Agency considers glycosylation status as part of the weight-of-evidence approach in evaluation of the allergenic potential of a protein. (Pedrosa *et al*., 2000; Wormald *et al*., 1999; Bencúrová *et al*., 2004).

Characterization studies conducted on both the DP23211 maize-derived and microbially-expressed IPD072Aa protein demonstrated that the IPD072Aa protein is not glycosylated.

*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 is included in the weight-of-evidence arguments in assessment of their allergenicity (Codex Alimentarius, 2003).

A study assessing the bioactivity of IPD072Aa after heat treatment found that the protein is heat stable up to 95 ℃, after which the protein loses its stability. Because corn products are typically not consumed raw and are often boiled, roasted, baked, or fried at temperatures of 100 ℃ or higher, it is expected that the IPD072Aa protein would become denatured during these processes.

## B. DvSSJ1 dsRNA

The hazard analysis for DvSSJ1 was examined via a "weight of evidence" approach using the mode of action, bioinformatics analysis, and physiological barriers in vertebrates. Based on the analysis below, DvSSJ1 represents a negligible risk to humans or livestock that consume DP23211 maize.

*Mode of action*
DvSSJ1 dsRNA is a double stranded RNA designed to target and down-regulate expression of DvSSJ1 protein found in the mid-gut of Western corn rootworm. DvSSJ1 dsRNA exerts its function through RNA interference (RNAi), a naturally occurring gene regulation mechanism that is ubiquitous in most plants and animals. DvSSJ1 dsRNA is highly specific to the sequence of the smooth septate junction protein 1 (*dvssj1*) gene specifically from WCR, a protein unique to invertebrates. This specificity lowers the likelihood that DvSSJ1 would pose a hazard to humans or livestock that consume DP23211 maize.

*Bioinformatics*
To identify the potential for DvSSJ1 dsRNA to induce off-target effects *in silico*, a study comparing the DvSSJ1 sense and antisense sequences to the human transcriptome was conducted. While this study did not identify any exact matches between DVSSJ1 dsRNA 21-mers and human transcripts, the results of this study are limited as the search threshold should have allowed for 0-2 mismatches between sequences. However, when combined with the low expression values in DP23211 maize, specificity of the dsRNA, and history of safe exposure to RNA as discussed in the human health waivers, the results from this study suggest that potential off-target effects in mammals as a result of consuming DP23211 maize are unlikely.

*Physiological barriers in humans and other vertebrates*
In addition to the specificity of DvSSJ1 as shown through bioinformatics and non-target organism bioassays resulting in an unlikely hazard, 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 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 DvSSJ1 poses no hazard to humans.

# IV. Human Exposure and Risk Characterization Assessment

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

### 1. Toxicological Profile and Allergenicity Assessment Conclusions

#### a. IPD072Aa Protein

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 the IPD072Aa protein 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, the IPD072Aa protein represents a negligible risk to human or livestock that consume maize products.

#### b. DvSSJ1 dsRNA

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 has determined that the DvSSJ1 dsRNA meets the definition of a nucleic acid residue in a PIP and confirms that this established exemption is applicable to the DvSSJ1 dsRNA expressed in DP23211 maize.

#### c. Inert PIPs PMI and PAT

DP23211 maize contains the inert PIPs phosphomannose isomerase (PMI) and phosphinothricin acetyltransferase (PAT). Based on sequence similarity, EPA has determined that the PMI and PAT proteins in event DP23211 are sufficiently similar to the proteins for which tolerance exemptions were granted and therefore are covered by the existing tolerance exemptions as stated in 40 CFR § 174.527 and 40 CFR § 174.522, respectively (USEPA 2023).

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

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 were observed in toxicological tests with the IPD072Aa protein as expressed in DP23211 maize as described previously; therefore, the EPA did not conduct a quantitative exposure assessment.

### a. Food Exposure

The proposed exemption from the requirement of a tolerance for residues of the IPD072Aa protein applies to the food and feed commodities of corn: corn, field; corn, sweet; and corn, pop. Grain serves as a basis for many commodities consumed by humans, thus dietary exposure to IPD072Aa protein is expected. As mentioned above, the highest mean concentration was found in root and was measured to be 42 ng/mg-dw. For context, the acute oral toxicity study found the $LD_{50}$ of IPD072Aa to be >2000 mg/kg bw in male and female mice. These values indicate that food exposure would be unlikely to surpass levels of IPD072Aa protein which were tested in the acute oral toxicity study and not toxic at that level. Further, IPD072Aa is rapidly digested in SGF and completely digested in SIF and bioinformatics analyses did not indicate a toxigenic or allergenic potential *in silico*. Therefore, as described in the section above, the IPD072Aa protein does not exhibit any mammalian toxicity via the oral route of consumption, and it also presents a minimal risk of being an allergen.

### b. Drinking Water Exposure

A quantitative drinking water exposure and risk assessment has not been conducted because drinking water exposure to residues of the active ingredient are expected to be negligible. A soil dissipation study found that the estimated dissipation time of IPD072Aa in various soils (loam, sandy clay loam, and silt loam) was within 7 days, it is therefore expected that biological processes will reduce run-off and potential exposure of drinking water to negligible levels.

Proteases and nucleases found in water and the environment would likely degrade the biological material containing the active ingredients, as they have been demonstrated to be susceptible to degradation through these means. Further, given the treatment process for municipal water plants (chemical addition, coagulation and flocculation, sedimentation and clarification, filtration, and disinfection) it is likely that IPD072Aa residues would be removed during the water treatment process (https://www.cdc.gov/healthywater/drinking/public/water_treatment.html). If exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity observed for IPD072Aa protein.

### c. Non-Occupational and Residential Exposure

Non-dietary residential exposure via inhalation is not likely. Corn is pollinated by wind, so it is possible that air in residential areas will carry transgenic corn pollen; however, corn pollen is not respirable, as it consists of spherical particles ranging in size from 80 to 125 µm (Hofmann et al., 2014), in contrast with respirable particles that are less than 10 µm. Inhalation exposure from sources other than pollen is not likely. IPD072Aa protein is contained within plant cells, which essentially eliminates non-occupational and residential inhalation exposure route or reduces it to negligible levels.

Non-dietary exposure via the skin is somewhat more likely via the contact with corn products which might have been processed in a way that disrupts cellular structure. The most likely way the proteins can have an effect via dermal exposure is by eliciting an allergic reaction (Barata and Conde-Salazar, 2013). The weight-of-evidence arguments in the section above support the lack of allergenicity for the IPD072Aa protein. Additionally, there exists a large number of proteases on the surface of the human skin (Stewart-McGuinness et al., 2022). These proteases will contribute to degradation of any proteins coming in contact with the skin. The EPA concludes that there will be no risks associated with dermal exposure to IPD072Aa.

## 3. Cumulative Effects

Section 408(b)(2)(D)(v) of FFDCA requires that, when considering whether to establish, modify, or revoke a tolerance, the Agency consider "available information" concerning the cumulative effects of a particular pesticide's residues and "other substances that have a common mechanism of toxicity." No risk of cumulative toxicity/effects from the IPD072Aa protein have been identified as no toxicity has been shown in the submitted studies. Therefore, EPA has not assumed that the IPD072Aa protein has a common mechanism of toxicity with other substances.

Based on the results of the acute oral toxicity study for the IPD072Aa protein, there is no indication of mammalian toxicity resulting from the plant-incorporated protectant IPD072Aa as found in DP23211 maize. In the absence of such effects, we conclude that there are no identifiable cumulative effects for the IPD072Aa proteins.

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

### a. U.S. Population

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 IPD072Aa. This includes all anticipated dietary exposures and all other exposures for which there is reliable information.

### b. Infants and Children

For all of the reasons discussed previously, EPA has concluded that IPD072Aa is 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.

## A. Occupational Exposure and Risk Characterization

Dermal or pulmonary exposure to IPD072Aa is not likely as the PIP is contained within plant cells, which reduces these exposure routes to negligible levels. Worker exposure to the IPD072Aa protein via seed dust is also expected to be negligible due to the low amount of protein expressed in transformed plants. If such exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity.

## B. Residue Analytical Methods

The applicant submitted a protocol for a lateral flow test strip kit to be used for the detection of IPD072Aa protein in maize grain samples. The protocol adequately describes the methodology; however, no data were provided to confirm the specificity and sensitivity of this method. The applicant is currently in the process of having the test kit certified by USDA's Grain Inspectors, Packers and Stockyards Administration (GIPSA) and has requested that the availability of a validated lateral flow test kit be a condition of registration.

# V. Human Health Risk Assessment Conclusion

## A. IPD072Aa Protein

Based on the molecular characterization, protein expression, bioactivity, acute oral toxicity, lack of glycosylation, complete digestion in both SGF and SIF, and bioinformatics, the weight of evidence suggests that the IPD072Aa protein, and the genetic material necessary for its production in DP23211 maize, is unlikely to cause an adverse effect on humans when exposed via the oral route.

EPA has determined that there is reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the IPD072Aa protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because, as previously discussed, no toxicity to mammals has been observed, nor any indication of allergenicity potential for this plant-incorporated protectant.

## B. DvSSJ1 dsRNA

Based on the bioactivity data, bioinformatics studies, and human health waivers, the weight of evidence suggests that DvSSJ1, and the genetic material necessary for its production in DP23211 maize, is unlikely to cause an adverse effect on humans. 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 DvSSJ1 dsRNA expressed in DP23211 maize, indicating a negligible risk to human or livestock that consume maize products.

# VI. References

Arrebola E., Tienda S., Vida C., de Vicente A., Cazorla F., (2019). Fitness Features Involved in the Biocontrol Interaction of Pseudomonas chlororaphis With Host Plants: The Case Study of PcPCL1606. Frontiers in Microbiology. doi:10.3389/fmicb.2019.00719

Barata AR, Conde-Salazar L. Protein contact dermatitis--case report. *An Bras Dermatol*. 2013;88(4):611-613. doi:10.1590/abd1806-4841.20132023

Besler M, Steinhart H, Paschke A. 2001. Stability of food allergens and allergenicity of processed foods. J Chromatogr B Biomed Sci Appl.; 756:207–228.

Chin-A-Woeng T., Thomas-Oates J., Lugtenberg B., Bloemberg G., (2001). Introduction of the phzH Gene of Pseudomonas chlororaphis PCL1391 Extends the Range of Biocontrol Ability of Phenazine-1-Carboxylic Acid-Producing Pseudomonas spp. Strains. MPMI (14) 8. pp. 1006–1015.

Codex Alimentarious Commission, 2003. Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarious Commission, Twenty-Fifth Session, Rome, Italy, 30 June–5 July, 2003. Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant DNA plants, and Appendix IV, Annex on the assessment of possible allergenicity, pp. 47–60.

Furuse M, Izumi Y. Molecular dissection of smooth septate junctions: understanding their roles in arthropod physiology. Ann N Y Acad Sci. 2017 Jun;1397(1):17-24. doi: 10.1111/nyas.13366. PMID: 28636800.

Hofmann, F., Otto, M. & Wosniok, W. Maize pollen deposition in relation to distance from the nearest pollen source under common cultivation - results of 10 years of monitoring (2001 to 2010). Environ Sci Eur 26, 24 (2014).

Huang, H., Davis, C. D., & Wang, T. T. Y. (2018, Feb 15). Extensive Degradation and Low Bioavailability of Orally Consumed Corn miRNAs in Mice. Nutrients, 10(2). https://doi.org/10.3390/nu10020215

Hu X, Richtman NM, Zhao J-Z, Duncan KE, Niu X, Procyk LA, Oneal MA, Kernodle BM, Steimel JP, Crane VC, Sandahl G, Ritland JL, Howard RJ, Presnail JK, Lu AL, Wu G (2016) Discovery of midgut genes for the RNA interference control of corn rootworm. Scientific Reports 6: 30542

Pedrosa, C., De Felice, F.G., Trisciuzzi, C. and Ferreira, S.T., 2000. Selective neoglycosylation increases the structural stability of vicilin, the 7S storage globulin from pea seeds. *Archives of Biochemistry and Biophysics*, *382*(2), pp.203-210.

Pekar J, Ret D, Untersmayr E. Stability of allergens. Mol Immunol. 2018 Aug;100:14-20. doi: 10.1016/j.molimm.2018.03.017. Epub 2018 Mar 30. PMID: 29606336; PMCID: PMC6020993.

Petrick JS, Brower-Toland B, Jackson AL, Kier LD. Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: a scientific review. Regul Toxicol Pharmacol. 2013 Jul;66(2):167-76. doi: 10.1016/j.yrtph.2013.03.008. Epub 2013 Apr 2. PMID: 23557984.

Rodrigues, T. B., & Petrick, J. S. (2020). Safety Considerations for Humans and Other Vertebrates Regarding Agricultural Uses of Externally Applied RNA Molecules. Front Plant Sci, 11, 407. https://doi.org/10.3389/fpls.2020.00407

Schellenberger U, Oral J, Rosen BA, Wei J-Z, Zhu G, Xie W, McDonald MJ, Cerf DC, Diehn SH, Crane VC, Sandahl GA, Zhao J-Z, Nowatzki TM, Sethi A, Liu L, Pan Z, Wang Y, Lu AL,

Wu G, Liu L (2016). A selective insecticidal protein from Pseudomonas for controlling corn rootworms. Science 354: 634-637

Shreffler, W.G., Castro, R.R., Kucuk, Z.Y., Charlop-Powers, Z., Grishina, G., Yoo, S., Burks, A.W. and Sampson, H.A., 2006. The major glycoprotein allergen from Arachis hypogaea, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant in vitro. *The Journal of Immunology*, *177*(6), pp.3677-3685.

Stewart-McGuinness, C.; Platt, C.I.; Ozols, M.; Goh, B.; Griffiths, T.W.; Sherratt, M.J. Defining the Protease and Protease Inhibitor (P/PI) Proteomes of Healthy and Diseased Human Skin by Modified Systematic Review. Biomolecules 2022, 12, 475. https://doi.org/10.3390/biom12030475

Torri, A., Jaeger, J., Pradeu, T. and Saleh, M.C., 2022. The origin of RNA interference: Adaptive or neutral evolution?. Plos Biology, 20(6), p.e3001715.

USEPA. 2023. Data Evaluation Record for Equivalency Determination for the PMI and PAT inert Plant Incorporated Protectants in Event DP23211 Corn (EPA File Symbol: 29964-ET). Memo from Nina Ortiz to Amanda Pierce, dated September 26, 2023.

USEPA. 2024. Ecological Effects and Environmental Fate Assessment for the Plant-Incorporated Protectants IPD072Aa and DvSSJ1 and the Genetic Material Necessary for their Production in DP23211 Maize. Data and Information Were Provided in Support of an Application for a FIFRA Section 3 Seed Increase Registration. EPA File Symbol: 29964-ET. Memo from Kara Welch to Mike Mendelsohn thru Amanda Pierce, dated January 19, 2024.

Wormald, M.R. and Dwek, R.A., 1999. Glycoproteins: glycan presentation and protein-fold stability. *Structure*, *7*(7), pp. R155-R160.