Novel food information: Insect resistant and herbicide tolerant DP-023211-2 Maize

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Background:

Health Canada has notified Pioneer Hi-Bred Canada Company that it has no objection to the food use of insect resistance and herbicide tolerant maize line DP-023211-2 (hereafter reffered as DP23211). The Department conducted a comprehensive safety assessment of this maize line according to its Guidelines for the Safety Assessment of Novel Foods. These Guidelines are based upon internationally accepted principles for establishing the safety of foods with novel traits.

The following provides a summary of the notification from Pioneer Hi-Bred Canada Company and the evaluation by Health Canada. This document contains no confidential business information.

1. Introduction

Pioneer Hi-Bred Canada Company has developed a genetically modified (GM) maize line (*Zea mays L.*), DP23211, that exhibits resistance to western corn rootworm (*Diabrotica virgifera virgifera*) (WCR) and tolerance to glufosinate-ammonium herbicide. The insect resistance trait was achieved through expression of the DvSSJ1 double-stranded RNA (dsRNA) and the IPD072Aa protein. The herbicide tolerant trait was achieved through expression of the maize-optimized phosphinothricin acetyltransferase (mo-PAT) protein. In addition, the phosphomannose isomerase (PMI) protein was used as a selection marker.

The expression of the insecticidal IPD072Aa protein, encoded by the *ipd072Aa* gene, disrupts the gut epithelium of WCR and results in larval death. The expression of the DvSSJ1 double-stranded RNA (dsRNA) interferes with the production of the WCR gut lining protein DvSSJ and results in larval mortality. The expression of the maize-optimized phosphinothricin acetyltransferase (mo-PAT) protein allows tolerance to glufosinate herbicide. Expression of the phosphomannose isomerase (PMI) protein was used as a selection marker. The *pmi* gene has been previously assessed by Health Canada in insect resistant maize event MIR 162 (2010).

The safety assessment performed by the Food Directorate evaluators was conducted according to Health Canada's *Guidelines for the Safety Assessment of Novel Foods*. These Guidelines are based on harmonization efforts with other regulatory authorities and reflect international guidance documents in this area (e.g., Codex Alimentarius). The assessment considered: how western corn rootworm resistant and herbicide tolerant maize DP23211 was developed, how the composition and nutritional safety of this variety compared to its unmodified comparator, and what the potential is for this variety to present a toxic or allergenic concern. Pioneer Hi-Bred Canada Company has provided data to support that this variety is safe for use as food in Canada.

The Food Directorate has a legislated responsibility for the pre-market assessment of novel foods and novel food ingredients, as detailed in Division 28 of Part B of the *Food and Drug Regulations (Novel Foods)*. Western corn rootworm resistant and glufosinate-tolerant maize DP23211 is considered to be a novel food under the following part of the definition of novel foods:

> “c) a food that is derived from a plant, animal, or microorganism that has been genetically modified such that
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> i. the plant, animal or microorganism exhibits characteristics that were not previously observed in that plant, animal or microorganism”.

2. Development of the modified plant

The development of DP23211 maize occurred through two sequential transformation steps leading to the integration of the four expression cassettes (i.e., *pmi*, *mo-pat*, *ipd072Aa* genes and DvSSj1 dsRNA fragments) in the host genome (i.e., elite-inbred maize line PHR03). The first step consisted of the insertion of an integration site sequence (present in plasmid PHP56614 and referred to as the landing pad) between the two native zm-SEQ8 and zm-SEQ9 sequences, which appear in tandem in the receiving PHR03 maize line genome. This was achieved by microprojectile bombardment and the use of the I-CREI endonuclease. As a second transformation step, integration of notified expression cassettes was performed by *Agrobacterium*-mediated transformation of the selected maize line carrying the landing pad with plasmid PHP74643. This latter plasmid contains all four notified expression cassettes

During the transformation, *Agrobacterium tumefaciens* virulence genes located on the backbone of plasmid PHP74643 allowed the migration of the plasmid into the plant cell nucleus while integration of the notified expression cassettes at the landing pad occurred via flippase recombinase (encoded by a mo-flp expression cassette located on plasmid PHP74643). Plasmid PHP74643 also contained the

DsRed2 expression cassette encoding a tissue-specific protein, and the WUS and ODP2 proteins (encoded by *zm-wus2* and *zm-odp2* genes) allowing improved regeneration of the transformed maize plant.

The cloning strategy and the design of the construct prevented the virulence genes, I-CREI, *zm-wus2*, *zm-odp2*, *mo-flp*, and *DsRed2* expression cassettes from being integrated in the maize genome while allowing transient expression of their gene products. Transformed seeds were sorted based on the tissue-specific expression of DsRed2 protein in the aleurone layer which produced a transient red fluorescent coloration. Following both transformation steps, the T0 plant tissues were grown in tissue culture using mannose as carbon source to select for the expression of *pmi* gene.

The coding sequences for the IPD072Aa, PAT, and PMI proteins were obtained from the genomic DNA of *Pseudomonas chlororaphis* strain SS143D5, *Streptomyces viridochromogenes* strain Tü494, and *Escherichia coli* K-12, respectively.

The DvSSJ1 dsRNA was designed to be complementary to the *dvssj1* mRNA, coding a specific portion of the WCR DvSSJ1 protein. Two inverted complementary DvSSJ1 fragments are contained in the DvSSj1 dsRNA expression cassette and each fragment is flanked by stop codon sequences to terminate translation through the site. An intron connector was inserted between the two DvSSJ1 fragments to allow formation of the dsRNA via a hairpin shape which is obtained by complementarity of the fragments. The notified DvSSJ1 dsRNA was isolated from genomic WCR neonates and cloned in a vector. The molecular target of DvSSJ1 dsRNA is arthropod-specific, has not been described in

vertebrates, and has been shown to be specific to the *Diabrotica* species. The petitioner provided a literature review supporting history of safe use of dsRNA found in commonly consumed plants with sequences complementary to human genes.

The petitioner provided information to support the safety and historical use of each donor organism and the recipient organism (i.e., elite-inbred line PHR03). None of these organisms poses a health or safety concern.

3. Characterization of the modified plant

Genomic DNA from leaf tissues of DP23211 maize was analyzed by Southern-by-Sequencing (SbS) to estimate the number of insertion sites, verify insert intactness, identify plasmid-plasmid or plasmid-genome junction sequences that occurred due to the inserted DNA, and confirm the absence of plasmid backbone sequences and unintended plasmid-derived integration(s).

Results of the SbS analysis demonstrated the presence of a single, intact copy of the insert located at the intended location in the DP23211 maize genome. Analysis of both junction sequences demonstrated that there was no partial or total deletion of any endogenous gene(s), including the two targeted native zm-SEQ8 and zm-SEQ9 sequences. Results of the SbS analysis also demonstrated absence of plasmid-derived off-target insertions and absence of any plasmid backbone sequences.

All plasmids employed in the development of DP23211 maize contained antibiotic resistance genes in their backbone. The petitioner showed the absence of antibiotic resistance genes in the DP23211 maize genome through the absence of plasmid backbone sequences by SbS analysis.

A bioinformatics assessment of translated open-reading frames (ORFs) was performed on the insertion site and border junctions of DP23211 maize for homology to known and putative allergens and toxins following established international criteria [1] [2]. ORFs of lengths equal to or higher than 30 amino acids were considered. Putative translated ORFs were searched against the Comprehensive Protein Allergen Resource (COMPARE) 2019 database [3] and the Pioneer toxin database. None of the putative translated ORFs at the DP23211 maize insertion site and border junctions returned alignments from the search against both databases.

Stability of each expression cassette in DP23211 maize genome was demonstrated by assessing individual DP23211 maize from five generations (T1, T2, T3, T4, and T5) by means of Southern blot analysis. Hybridization of all probes resulted in a single band of the expected size in all five generations of DP23211 maize samples analysed. Hybridization patterns exhibited event-specific bands unique to the DP23211 maize insertion and therefore demonstrated the genetic stability of this event across all tested generations.

Genomic DNA from individual plants of five DP23211 maize generations were tested for the presence/absence of the DP23211 insertion and the following genes/fragments using qPCR: *ipd072Aa*, *mo-pat*, *pmi*, and DvSSJ1. The probes employed were specific to the event and the copy number for each plant was calculated based on use of a known copy number calibrator (i.e., taxon reference hmg-A) and the application of the 2-ΔΔCT method to differentiate 1 and 2 copy samples [4]. Also, a phenotypic analysis evaluated the tolerance to glufosinate-ammonium for each individual plant and phenotypic results were compared to the qPCR results to verify co-segregation of genotype with phenotype.

Segregation ratios determined for the 5 generations of the maize event DP23211 demonstrated that *ipd072Aa*, *mo-pat*, *pmi* genes, the DvSSJ1fragments, and the maize event DP23211 as a whole segregated together and in accordance with the Mendelian rule of inheritance for a single genetic locus. In addition, these results support that the DNA insertion co-segregated with the phenotype trait and was stable through traditional breeding.

4. Product information

Event DP23211 differs from its traditional counterparts by the expression of PAT, PMI, IPD072Aa proteins, and the DvSSJ1 dsRNA. Indirect ELISA and QuantiGene Plex Assay were used to measure expression levels of introduced proteins and dsRNA, respectively, in plant tissues (leaf, root, pollen, forage, whole plant, and grain samples) through a field trial. The field trial was a randomized complete block design conducted in 2018 at seven sites in the US (two sites in Illinois, one site in each Iowa, Indiana, Minnesota, Pennsylvania, and Texas) and one site in Canada (Ontario) with four blocks at each site. All samples were collected from impartially selected, healthy, representative plants to minimize potential bias.

In addition to the analysis of different plant tissues, the company assessed the expression of notified proteins and dsRNA in different maize growth stages, which are described by Abendroth et al. (2011) [5]. None of the notified proteins or dsRNA were expected to be expressed in a tissue-specific manner since all promotors that drive the transcriptional regulation of notified proteins or dsRNA are not tissue-specific. The DsRed2 expression cassette was not integrated into the genome and its expression was transient.

The average level of DvSSJ1 dsRNA in plant tissues varied between 9.87×10^{-4} to 6.46×10^{-2} µg/g of tissue dry weight. At maturity stage (R6), leaves (1.32×10^{-2} µg/g of tissue dry weight), roots (1.15×10^{-2} µg/g tissue dry weight), and whole plant samples (1.08×10^{-2} µg/g of tissue dry weight) contained similar concentrations of DvSSJ1 dsRNA. The matured grain tissues contained an average of 4.13×10^{-3} µg/g of tissue dry weight.

The average level of IPD072Aa protein in plant tissues varied between 0.65 to 31 ηg/g of tissue dry weight. At maturity, the roots contained the most amount of this protein (31 ηg/g of tissue dry weight). This observation is logical since IPD072Aa protein aims to confer resistance to western corn rootworm, which mainly attacks the plant root system. At maturity stage, the grains contained an average of 2.1 ηg/g of tissue dry weight. Within all plant tissues, the expression level of IPD072Aa protein remained consistent across all growth phases tested, except for leaf tissue at maturity stage that decreased drastically. Expression levels of IPD072Aa protein in plant tissues were as predicted.

The average level of PAT protein in plant tissues varied between <0.11 to 58 ηg/g of tissue dry weight. At maturity stage, the grains contained an average of 5.1 ηg/g of tissue dry weight. Among all plant tissues tested at different growth stages, PAT expression levels decreased as growth stages advanced. All samples (24) tested in leaves at maturity stage exhibited values below the lower limit of quantification (LLOQ). Since the expression levels decreased as growth stages advanced, such results suggest that leaves contain virtually no PAT protein. Expression levels of PAT protein in plant tissues are not a safety concern.

The average level of PMI protein in plant tissues varied between <0.3 to 9.4 ηg/g of tissue dry weight. At maturity stage, the grains contained an average of 4.3 ηg/g of tissue dry weight. In roots, the expression level of PMI protein decreased as growth stages advanced. Expression levels of PMI protein in plant tissues are not a safety concern.

The notified PMI, PAT, IPD072Aa proteins are 391, 183, and 86 amino acids in length, respectively, as well as a molecular weight of approximatively 43, 21, and 10 kDa. The petitioner provided Western blot analysis that showed a single predominant band at the expected molecular weight and demonstrated immunoreactivity using PMI and PAT monoclonal antibodies. With regard to IPD072Aa protein, a Western blot analysis using IDP072Aa polyclonal antibodies showed a single band with the expected molecular weight. Also, IPD072Aa protein migrated to the expected molecular weight in a SDS-PAGE analysis.

Based on the information provided, there are no concerns regarding the food use of DP-23211 maize from a molecular perspective.

5. Dietary exposure

It is expected that DP23211 maize will be used in applications similar to conventional maize varieties. The petitioner does not anticipate a significant change in the food use of maize with the introduction of DP23211 maize.

6. Nutrition

The nutritional and anti-nutritional components of the DP23211 maize were analyzed and compared to a non-genetically modified (non-GM), near isoline maize conventional control. This was done as part of the field trial described above where each block contained DP23211 maize, control maize, and non-GM commercial maize reference lines. The petitioner also notes that reference maize and control maize samples were collected prior to collection of DP23211 maize samples to minimize the potential for cross contamination.

The compositional analytes measured in DP23211 and non-GM control maize were: proximates, fatty acids, amino acids, minerals, vitamins, secondary metabolites, and antinutrients. The analytes included those recommended by the Organisation for Economic Co-operation and Development (OECD) Consensus document on compositional considerations for new varieties of maize (OECD, 2002).

Of the analytes measured, statistically significant differences were noted in the following analytes tested in DP23211 compared to control: oleic acid (lower in DP23211 than control), arachidic acid (higher), eicosenoic acid (higher), vitamin B6 (lower), alphatocopherol (lower), and p-coumaric acid (lower). The magnitude of difference between DP23211 and the control was small and unlikely to impact Canadian dietary intakes. In addition, the values were within OECD ranges, tolerance intervals, reference ranges and/or literature ranges.

The petitioner has demonstrated that DP23211 maize has similar nutritional composition to its control. Based on the information provided, no nutritional concerns were found with the sale of foods derived from DP23211 maize in Canada.

7. Chemistry

Data for toxic trace elements were not provided by the petitioner. However, the petitioner provided the results of a compositional analysis of DP23211 maize in comparison to its non-genetically modified (GM) counterpart and reference varieties. Maize samples were assessed for calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc. The petitioner concluded that there were no statistically significant differences the mineral levels between the DP23211 maize and the control. This conclusion was supported by the Bureau of Nutritional Sciences and suggests that there would be no indication that the genetic modifications would result in significant differences in the uptake of toxic trace elements in DP23211 maize relative to conventional maize.

Data for mycotoxins were also not provided by the petitioner. However, a qualitative assessment was conducted comparing responses to naturally occurring insects, diseases and stressors for DP23211 maize, its non-GM counterpart and reference varieties. Information in the submission indicates that DP23211 maize did not exhibit any major differences in responses to naturally occurring insects or diseases and abiotic stressors. Since infection from pests, disease and abiotic stressors can increase the susceptibility of a plant to fungi producing mycotoxins, it can be inferred that the modifications to DP23211 maize are unlikely to increase its susceptibility to mycotoxins compared to conventional varieties of maize.

Data were provided by the petitioner regarding agronomic characteristics of DP23211 maize, its non-GM counterpart and reference varieties. The petitioner indicated in the submission that the majority of characteristics measured in the various types of maize as part of the agronomic evaluation did not

demonstrate any statistically significant differences. For the few characteristics where a difference was observed between DP23211 maize and the control, the petitioner indicated that these were not biologically meaningful as they were well within the range of natural variation observed in other reference varieties. Increased uptake of chemical contaminants and greater susceptibility to mycotoxins would potentially impact plant growth and yield. The above information suggests that the genetic modification in DP23211 maize is not expected to have a negative impact on crop yield relative to other conventional maize varieties

DP23211 maize is highly comparable to conventional maize, which also suggests that it is unlikely that DP23211 maize would have an increased ability to uptake chemical contaminants or greater susceptibility to mycotoxin-producing fungi relative to non-GM maize.

Based on the information provided, DP23211 maize would not be expected to pose a concern from a chemical contaminants perspective.

8. Toxicology

No mortality was observed (LD_{50}>2000 mg/kg bw) in an acute oral toxicity study of the IPD072Aa protein produced in a microbial expression system. The study followed OECD guidelines and was compliant with Good Laboratory Practices (GLP). The petitioner provided scientific data that confirmed the equivalency of the microbially produced IPD072Aa protein and the protein produced in the maize through means of SDS-PAGE, Western blot, protein glucosylation analysis as well as peptide mapping by mass spectrometry (MS) and N-terminal amino acid sequencing.

Comparison of the estimated human exposure to IPD072Aa from maize consumption (6.2 µg/kg bw per day) to the LD_{50} (> 2000 mg/kg bw) resulted in a margin of exposure (MOE) of > 300 000. The dietary exposure estimate was based on measured values in the grain and is likely an overestimate, as it assumed that all maize products eaten would be derived from this variety and the levels of IPD072Aa would not decrease during food processing. The MOE was considered sufficient from a safety perspective.

Overall, no toxicological concerns were identified for the IPD072Aa protein.

DvSSJ1 dsRNA is arthropod specific and has not been identified in vertebrates. No homology was observed between the small interfering RNA (siRNA) sequences involved in the DvSSJ1 RNA interference (RNAi) and mammalian RNA, including human RNA, suggesting that there would be no RNA in human cells which could be targeted by DvSSJ1 RNAi.

In addition, the numbers of dsRNA potentially reaching human cells after ingestion of the maize would be too low to result in RNAi (<1 copy/cell) in human cells. The dietary exposure estimate was based on measured values in the grain and is likely an overestimate as it assumes that all maize products eaten would be derived from this variety and the levels of dsRNA would not decrease during food processing.

It is expected that human gut epithelium, vascular endothelium and cellular membranes would present a significant physical barrier to absorption of dsRNA and nucleases in saliva, the gastrointestinal tract and the blood would degrade dsRNA before it could reach the cells.

Overall, no toxicological concerns have been identified for the DvSSJ1 dsRNA.

No mortality was observed (LD_{50}>5000 mg/kg bw) in an acute oral toxicity study of the PAT protein produced in a microbial expression system. The study followed OECD guidelines and was compliant with GLP. While the petitioner stated that the microbial PAT sequence was the same as that inserted into this maize, insufficient evidence was submitted to conclude on equivalency between the microbial and maize PAT protein. However, in lieu of this, Health Canada confirmed that the PAT protein in this petition is equivalent to the PAT protein discussed in Hérouet et al. (2005) [6]. This publication reported that no mortality was observed (LD_{50}>10 mg/kg bw) in an acute intravenous (i.v.) toxicity study of the PAT protein.

Comparison of the estimated human exposure (15.1 ug/kg bw per day) to the i.v. LD_{50} (>10 mg/kg bw) results in an MOE > 662 while comparison to the oral LD_{50} (> 5000 mg/kg bw) results in an MOE > 300 000. The exposure estimate was based on measured values in the grain and is likely an overestimate as it assumes that all maize products eaten would be derived from this variety and the levels of PAT would not decrease during food processing. The MOEs were considered sufficient from a safety perspective.

Overall, no toxicological concerns were identified for the PAT protein.

Health Canada (2010) has previously approved a maize line for food use which included the PMI protein used as a selectable marker (Syngenta Seeds Canada, MIR162). The PMI protein was found to have low acute oral toxicity (LD_{50}>3080 mg/kg bw). Pioneer submitted a letter of authorization from Syngenta stating that information on the PMI protein can be used for this maize product. The

petitioner provided data confirming equivalency between the previously approved PMI protein and the protein produced in DP23211 maize through means of sequence alignment and Western blot analysis.

Overall, no toxicological concerns were identified for the PMI protein.

Based on the information provided, DP23211 maize would be as safe as conventional maize in terms of potential toxicity.

9. Allergenicity

The amino acid sequence for IPD072Aa and PAT proteins were compared to the sequences for known or suspected allergens (Comprehensive Protein Allergen Resource (COMPARE), Jan., 2019: 2081 sequences). No matches were identified, suggesting that IPD072A and PAT proteins are unlikely to cross-react with known or suspected allergens.

IPD072Aa protein was completely digested within 0.5 minutes in a simulated gastric fluid (SGF) assay and completely digested within 20 minutes in a simulated intestinal fluid (SIF) assay. These data suggest that the protein would likely be quickly and completely digested in the human digestive tract, unlike some food allergens which are resistant to digestion.

Hérouet et al. (2005) presents evidence that the PAT protein is degraded quickly and completely (less than 0.5 minutes) in an SGF assay and in an SIF assay. These data suggest that the protein would likely be quickly and completely digested in the human digestive tract, unlike some food allergens which are resistant to digestion.

Overall, no allergenicity concerns were identified for IPD072A and PAT proteins.

Most food allergens are proteins. As the dsRNA acts through RNAi and does not produce a protein, it would not be expected to be a food allergen.

The amino acid sequence for PMI protein was compared to the sequences for known or suspected allergens (COMPARE, Jan., 2019: 2081 sequences). No sequence similarity greater than 35% over 80 or more amino acids was identified. No eight amino acid matches were identified, other than the previously identified eight amino acid match with *Rana* (frog). Subsequent negative results for IgE binding using the serum of the single individual known to have this allergy was considered in the previous evaluation (Health Canada, 2010). Overall, PMI protein is unlikely to cross-react with known or suspected allergens based on a lack of amino acid sequence homology and a lack of IgE binding in serum. In a previous Health Canada evaluation, the PMI protein was found to be readily degraded in an SGF assay and inactivated by heat. These data suggest that the protein would likely be quickly and completely digested in the human digestive tract, unlike some food allergens which are resistant to digestion.

Overall, no allergenicity concerns were identified for the PMI protein.

Based on the information provided, DP23211 maize would be as safe as conventional maize in terms of food allergenicity.

Conclusion:

Health Canada’s review of the information presented in support of the use of insect resistant and herbicide tolerant maize variety DP-023211-2 does not raise concerns related to food safety.

Health Canada's opinion refers only to the food use of DP-023211-2 maize. Issues related to its use as animal feed have been addressed separately through existing regulatory processes in the Canadian Food Inspection Agency.

This Novel Food Information document has been prepared to summarize the opinion regarding the subject product provided by the Food Directorate, Health Products and Food Branch, Health Canada. This opinion is based upon the comprehensive review of information submitted by the petitioner according to the Guidelines for the Safety Assessment of Novel Foods.

(Également disponible en français)

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Footnotes

1 Codex Alimentarius Commission (2003) Alinorm 03/34: Appendix III: Draft guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants, and Appendix IV: Proposed Draft Annex of the Assessment of Possible Allergenicity. Food and Agriculture Organization of the United Nations, World Health Organization, Rome, pp 47-60

2 FAO/WHO (2001) Evaluation of Allergencity of Genetically Modified Foods: Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 22 - 25 January 2001. Food and Agriculture Organization of the United Nations, Rome.

3 http://comparedatabase.org

4 Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. Methods 25: 402-408

5 Abendroth LJ, Elmore RW, Boyer MJ, Marlay SK (2011) Corn Growth and Development. Iowa State University Extension, PMR 1009

6 Hérouet, C., Esdaile, D.J., Mallyon, B.A., Debruyne, E., Schulz, A., Currier, T., Hendricks, K., Jan van der Klis, R. and Rouan, D. 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the pat and bar sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regulatory toxicology and pharmacology, 41: 134-149

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