Pioneer Petition (20-203-01p) for Determination of Non-regulated Status of DP23211 Maize

OECD Unique Identifier:
DP-∅23211-2

Draft Plant Pest Risk Assessment

January 2023

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A. Introduction

Pioneer Hi-Bred International, Inc. (hereafter referred to as Pioneer) has submitted a petition to the Animal and Plant Health Inspection Service (APHIS) of the United States Department Agriculture (USDA) seeking a determination of nonregulated status for maize (*Zea mays*) event with OECD Unique Identifier DP-∅23211-2 (hereafter referred toa s DP23211 maize) developed using genetic engineering for insect resistance and herbicide tolerance traits. The petition presents evidence supporting the argument that DP23211 maize is unlikely to pose a plant pest risk and, therefore, should no longer be regulated under the APHIS' 7 Code of Federal Regulations (CFR) part 340. This petition was assigned the number 20-203-01p and is hereafter referenced as Pioneer 2020 (Pioneer 2020). Under the authority of the plant pest provisions of the Plant Protection Act (7 U.S.C. 7701 et seq. 2020), the regulations in 7 CFR part 340, "Movement of Organisms Modified or Produced Through Genetic Engineering," regulate, among other things, the importation, interstate movement, or release into the environment of organisms modified or produced through genetic engineering that are plant pests or pose a plausible plant pest risk. This plant pest risk assessment (PPRA) was conducted to determine if DP23211 maize is unlikely to pose a plant pest risk.

The petition for a determination of nonregulated status described in this PPRA is being evaluated under the version of the regulations effective at the time the petition was received. APHIS issued a final rule, published in the Federal Register on May 18, 2020 (85 FR 29790-29838, Docket No. APHIS-2018-0034)[1], revising 7 CFR part 340. Since the petition for determination of nonregulated status for DP23211 was received by APHIS on July 21, 2020, before the revisions to the regulations became final, this petition request is being evaluated in accordance with the [legacy] regulations at 7 CFR 340.6 (e) (2020).

DP23211 maize was produced through use of *Agrobacterium tumefaciens* as a vector to transfer specific genetic sequences from plasmid PHP74643 (Pioneer 2020). Portions of the introduced genetic sequences in the T-DNA of plasmid PHP74643 come from plant pest organisms listed in 7 CFR § 340.2 (Table 5, p. 57-58, Pioneer 2020). Therefore, DP23211 maize is considered a regulated article under APHIS regulations at 7 CFR part 340 (7 CFR 340 2020). Pioneer has conducted field releases of DP23211 maize under APHIS authorizations since 2015 (Pioneer 2020), in part, to gather information to support that DP23211 maize is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived.

Potential impacts in this Plant Pest Risk Assessment (PPRA) are those that pertain to plant pest risk associated with DP23211 maize and its progeny and their use in the absence of confinement relative to the unmodified recipient and/or other appropriate comparators. APHIS utilizes data and information submitted by the applicant, in addition to current literature, to determine if DP23211 maize is unlikely to pose a plant pest risk. APHIS regulations in 7 CFR 340.6(c) (7 CFR 340 2020) specify the

[1] To view the final rule, go to www.regulations.gov and enter APHIS-2018-0034 in the Search field.

information needed for consideration in a petition for nonregulated status. APHIS assessed information submitted by the applicant about DP23211 maize related to: plant pest risk characteristics; expression of the gene product, new enzymes, or changes to plant metabolism; disease and pest susceptibilities and indirect plant pest effects on other agricultural products; effects of the regulated article on nontarget organisms; weediness of the regulated article; impact on the weediness of any other plant with which it can interbreed; changes to agricultural or cultivation practices that may impact diseases and pests of plants; and transfer of genetic information to organisms with which it cannot interbreed.

APHIS may also consider information relevant to reviews conducted by other agencies that are part of the 'Coordinated Framework for the Regulation of Biotechnology' (51 FR 23302 1986; 57 FR 22984 1992). (57 FR 22984 1992) (57 FR 22984 1992) (57 FR 22984 1992) Under the Coordinated Framework, the oversight of biotechnology-derived plants rests with APHIS, the Food and Drug Administration (FDA), and the Office of Pesticide Programs of the U.S. Environmental Protection Agency (EPA). Depending on its characteristics, certain biotechnology-derived products are subjected to review by one or more of these agencies.

Under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136 *et seq.*), EPA regulates the distribution, sale, use and testing of pesticidal substances produced in plants and microbes, including those pesticides that are produced by an organism through techniques of modern biotechnology. EPA also sets tolerance limits for residues of pesticides on and in food and animal feed, or establishes an exemption from the requirement for a tolerance, under the Federal Food, Drug and Cosmetic Act (FFDCA) (21 U.S.C. *et seq*. 2018 Edition). Prior to registration for a new use, or for a new or previously registered pesticide, EPA must determine through testing that the pesticide does not cause unreasonable adverse effects on humans, the environment, and non-target species when used in accordance with label instructions. EPA must also approve the language used on the pesticide label in accordance with Data Requirements for Pesticides (40 CFR part 158 2019 Edition). Other applicable EPA regulations include Pesticide Registration and Classification Procedures (40 CFR part 152 2019 Edition.), Procedures and Requirements for Plant Incorporated Protectants (PIPs) (40 CFR part 174 2019 Edition.), and Experimental Use Permits (40 CFR part 172 2019 Edition.). Tolerance petitions for IPD072Aa protein and DvSSJ1 RNAi were submitted in March 2020 (85 FR 17328) to the U.S. Biopesticides and Pollution Prevention Division (BPPD) under Section 3 of FIFRA.

The FDA under the FFDCA is responsible for ensuring the safety and proper labeling of all plant-derived foods and feeds, including those developed through modern biotechnology. To help sponsors of foods and feeds derived from crops developed using genetic engineering comply with their obligations, the FDA encourages them to participate in its voluntary early food safety evaluation for new non-pesticidal proteins produced by new plant varieties intended to be used as food (FDA, 2006), and a more comprehensive voluntary consultation process prior to commercial distribution of food or feed (57 FR 22984, 1992). Pioneer initiated a consultation with the FDA

(Biotechnology Notification File [BNF] No. 175) on the food and feed safety and compositional assessment of DP23211 maize on May 31, 2019. Pioneer received a completed consultation letter from the FDA on July 31, 2022. A copy of the text of this letter responding to BNF 175, as well as a copy of the text of FDA's memorandum summarizing the information in BNF 175, is available via the FDA webpage "New Plant Variety Consultations" at https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=NewPlantVarietyConsultations (US-FDA 2022).

B. Development of DP23211 maize

Maize (*Zea mays* ssp. *mays*), also commonly referred to as corn in English-speaking countries, is the most widely cultivated feed grain crop in the United States and the United States is the world's largest producer (FAOSTAT 2020; USDA-ERS 2020c). Maize is primarily grown for animal feed grain in the United States, accounting for more than 95% of 2019 total feed grain production, when over 14 billion bushels were produced on approximately 82 million acres (USDA-ERS 2020b, c). The average maize yield within the United States was an estimated 176 bushels per acre in 2020 (USDA-ERS 2020b).

To optimize yield and economic return, growers select maize lines adapted to local environmental and climatic conditions, growing them as annual row crops using appropriate cultivation practices (e.g., hybrid selection, seedbed preparation, planting timing and density, OECD 2003; Kansas State University 2007; University of Arkansas 2008; Nafziger 2009; NDSU 2019).

Maize productivity is impacted by losses due to abiotic factors (light, water, temperature, and nutrients) and biotic factors (weeds, pests, and pathogens). Plant pests can have a considerable influence on yield and productivity of crops; for example, total losses in maize due to biotic factors was estimated to be 31 - 38% between 1964 and 2003 (Oerke 2006). Average losses due to *Diabrotica* spp. infestations cost farmers approximately $2 billion in 2010(Wechsler and Smith 2018). Genetically engineered varieties with resistance to herbicides or insects are frequently employed to mitigate losses from weeds and pests in North America. In 2020, 92% of maize planted in North America was genetically engineered for herbicide resistance, pest resistance, or both (USDA-ERS 2020a).

DP23211 maize was developed by Pioneer to produce DvSSJ1 double-stranded ribonucleic acid (dsRNA) and the IPD072Aa protein for control of corn rootworm (CRW) pests, the phosphinothricin acetyltransferase (*mo-pat*) protein for tolerance to glufosinate-ammonium herbicides, and the phosphomannose isomerase (*pmi*) protein for use as a selectable marker. The genes were inserted into a proprietary Pioneer maize line via *Agrobacterium*-mediated transformation (Section I-B, p. 26, Pioneer 2020).

The DvSSJ1 suppression cassette produces an RNA transcript comprised of complementary inverted repeats derived from the coding sequence of the smooth septate junction protein 1 (*dvssj1*) from western corn rootworm (WCR; *Diabrotica virgifera virgifera*) (Hu et al. 2016), with intervening sequence for hairpin formation, and is intended to suppress translation of the DvSSJ1 protein via RNAi (Section III-A.1.4, p. 45, Pioneer 2020). The *ipd072Aa* cassette produces the insecticidal protein IPD072Aa, derived from *Pseudomonas chlororaphis*, which has shown activity against corn rootworm beetles (Schellenberger et al. 2016; Pioneer 2020). The IPD072Aa protein in DP23211 causes disruption of the midgut epithelium in susceptible insects (Section III-A.1.4, p. 45, Pioneer 2020). The *pmi* cassette produces the phosphomannose isomerase (PMI) protein derived from *Escherichia coli* (Negrotto et al. 2000), which is used as a selectable marker for plant transformation using mannose as the carbon source. Finally, the *mo-pat* cassette produces a maize-optimized version of phosphinothricin acetyltransferase (PAT) protein derived from *Streptomyces viridochromogenes* that confers resistance to glufosinate herbicides by deactivating phosphinothricin (Section III-A.1.4, p. 45, Pioneer 2020).

The proprietary Pioneer maize line that forms the genetic background of DP23211 maize was chosen as a recipient for transformation because it is both an elite line used for commercial products and is amenable to transformation (Section II-B, p. 30, Pioneer 2020). Several conventional Pioneer maize hybrid lines were used as comparators in field and safety assessments of DP23211 maize; these lines were chosen to represent a reference range of conventional hybrids grown in the Corn Belt (Section III-B.1, pp. 162-163, Pioneer 2020). Collectively, the near-isoline and commercial maize lines represent conventional controls that DP23211 maize can be compared to in field and safety assessments.

C. Description of Inserted Genetic Material, Its Inheritance and Expression, Gene Products, and Changes to Plant Metabolism

To inform the potential hazards resulting from the genetic modification and potential routes of exposure related to the inserted DNA and its expression products, APHIS assessed data and information presented in the petition related to: the transformation process; the source of the inserted genetic material and its function in both the donor organism and DP23211 maize; and the integrity, stability and mode of inheritance of the inserted genetic material through sexual or asexual reproduction based on the location of the insertion (e.g. nucleus or organelle) and the number of loci inserted.

APHIS also assessed data presented in the petition on whether the genetic modification results in expression of new genes, proteins, or enzymes or changes in plant metabolism or composition in DP23211 maize relative to conventional controls. The assessment encompasses a consideration of the expressed DvSSJ1 RNAi, and IPD072Aa, PMI and MO-PAT proteins and any observed or anticipated effects on composition of DP23211 maize, including any relevant changes in levels of metabolites, anti-nutrients, or nutrients

in grain and forage derived from DP23211 maize compared to those in the conventional controls.

This information is used later in this risk assessment to inform whether there is any potential for plant pest vectors or sequences to cause disease or greater plant pest risks in DP23211; or for expression of inserted DNA, new proteins or enzymes, or changes in metabolism to affect plant pest or diseases, nontarget beneficial organisms, weediness, agricultural practices that impact pest or diseases or their management, or plant pest risks through horizontal gene flow.

Description of the genetic modification and inheritance of inserted DNA

DP23211 maize was created by site-specific integration using two sequential transformation steps to (1) insert a specific integration site sequence (referred to as a "landing pad" sequence) at a specific location in the maize genome using Pioneer proprietary line PHR03 and (2) insert the intended expression cassettes from the plasmid PHP74643 T-DNA region into the landing pad (Section III-A.1, p. 31, Pioneer 2020).

The first transformation step utilized microprojectile co-bombardment with three plasmids to insert the landing pad into the PHR03 line using an I-CreI-endonuclease-mediated modification process (Section III-A.1.1, p 31, Pioneer 2020). Two plasmids were used solely to improve plant regeneration and were not incorporated into the maize genome; the third plasmid contained an excised I-CreI that is not incorporated into the maize genome, plus the landing pad. The I-CreI endonuclease was modified to create double-stranded breaks in the maize genome between sequences designated zm-SEQ8 and zm-SEQ9, where the landing pad was inserted via homology-directed repair. The landing pad consists of a maize ubiquitin promoter and 5'UTR that drives expression of an *nptII* marker gene that is flanked by flippase recombination sites. After transformation, regeneration of maize plants, and molecular characterization, a line with the landing pad and no unintended DNA was selected and advanced for the second step in the transformation process (Section III-A.1.1, p 32, Pioneer 2020).

The second transformation step utilized *Agrobacterium*-mediated transformation with the binary plasmid PHP74643 to transport the T-DNA into the plant cell nucleus and exchange the desired cassettes at the landing pad. The portion of the T-DNA that was not inserted into the maize genome included two cassettes for improved plant regeneration, a flippase recombinase, and a red fluorescent marker gene. The DsRed marker gene was used in this process to screen out plants with unintended DNA insertions (i.e. undesirable complete T-DNA integration into the genome). The transiently expressed flippase targets the recognition sites inserted in the first step of the process, resulting in excision of the nptII cassette and replacement with the desired cassettes. The intended insertion in DP23211 consists of the *pmi* and *mo-pat* cassettes, an intervening *Lox*P site, and the DvSSJ1 and *ipd072Aa* cassettes, respectively.

The intended T-DNA inserted into the maize line with landing pad in order to create DP23211 contains the following genetic elements[2] (Table 5, pp. 53-59, Pioneer 2020):

- Right Border: T-DNA Right Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al. 1996)
- *pmi*: Phosphomannose isomerase gene from *Escherichia coli* including 5' and 3' untranslated regions (UTR) (Negrotto et al. 2000)
- *pinII* Terminator: Terminator region from the Solanum tuberosum (potato) proteinase inhibitor II gene (Keil et al. 1986; An et al. 1989)
- *os*-actin Promoter: Promoter region from the *Oryza sativa* (rice) actin gene (GenBank accession CP018159; GenBank accession EU155408.1)
- *os*-actin Intron: Intron region from the *Oryza sativa* (rice) actin gene (GenBank accession CP018159; GenBank accession EU155408.1)
- *mo-pat*: Maize-optimized phosphinothricin acetyltransferase gene from *Streptomyces viridochromogenes* (Wohlleben et al. 1988)
- CaMV 35S Terminator: 35S terminator region from the cauliflower mosaic virus genome (Franck et al. 1980; Guilley et al. 1982)
- *ubi*ZM1 Promoter: Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen et al. 1992)
- *ubi*ZM1 5' UTR: 5' untranslated region from the *Zea mays* ubiquitin gene 1 (Christensen et al. 1992)
- *ubi*ZM1 Intron: Intron region from the *Zea mays* ubiquitin gene 1 (Christensen et al. 1992)
- All-stop Codon Sequence: DNA sequence containing stop codons to terminate translation in all six reading frames through the site
- DvSSJ1 Fragment: Fragment of the smooth septate junction protein 1 gene from *Diabrotica virgifera* (WCR) (Hu et al. 2016)
- Mini-stop Codon Sequence: DNA sequence containing stop codons to terminate translation in designated reading frames through the site
- *zm-Adh1* Intron Connector: Connector sequence derived from the intron 1 region of the *Zea mays* alcohol dehydrogenase gene (Dennis et al. 1984)
- Mini-stop Codon Sequence: DNA sequence containing stop codons to terminate translation in designated reading frames through the site

[2] Various short intervening sequences are present in the T-DNA of PHP74643 to facilitate cloning (Table 5, pp. 53-59, Pioneer 2020); however, these short intervening sequences are not included in the description of the T-DNA within the text. Additionally, several recombination sites to facilitate cloning are also present in the T-DNA of PHP74643 (Section III.A.1.4, p. 46 and Table 5, pp. 53-59, Pioneer 2020). These recombination sites are also not included in the description of the T-DNA within the text. These recombination sites include two flippase (Flp) sites, FRT1/FRT87; one loxP site; and four attB sites, attB1/attB2 and attB3/attB4. The presence of these sites does not cause recombination in the absence of a suitable recombinase enzyme; these recombinases are not naturally present in plants. These recombination sites are also not included in the description of the T-DNA within the text.

- DvSSJ1 Fragment: Fragment of the smooth septate junction protein 1 gene from *Diabrotica virgifera* (WCR) (Hu et al. 2016)
- All-stop Codon Sequence: DNA sequence containing stop codons to terminate translation in all six reading frames through the site
- Z27G Terminator: Terminator region from the *Zea mays* W64 line 27-kDa gamma zein gene (Das et al. 1991; Liu et al. 2016)
- BSV(AY) Promoter: Promoter region from the banana streak virus (acuminate Yunnan strain) genome (GenBank accession DQ092436.1; (Zhuang et al. 2011)
- *zm*-HPLV9 Intron: Intron region from the *Zea mays* predicted calmodulin 5 gene (Phytozome gene ID Zm00008a029682)
- *ipd072Aa*: Insecticidal protein gene from *Pseudomonas chlororaphis* (Schellenberger et al. 2016)
- at-T9 Terminator: Terminator region from an *Arabidopsis thaliana* putative gene of the mannose-binding protein superfamily (GenBank accession NM_001202984) (Salanoubat et al. 2000)
- Ti Plasmid Region: Sequence from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al. 1996)
- Left Border (LB): T-DNA Left Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al. 1996)

Pioneer confirmed the insertion and stability of the genetic elements listed above by conducting a detailed molecular characterization of the inserted T-DNA in DP23211 maize. An initial Southern-by-Sequencing analysis (SbSTM technology, hereafter referred to as SbS; see (Zastrow-Hayes et al. 2015) was utilized to determine the copy number and complexity of the T-DNA insertion, and the absence/presence of PHP74643 plasmid backbone sequences in DP23211 maize (Section V, p. 60, Pioneer 2020).

Additionally, Southern blot analysis and PCR/herbicide screening was utilized to determine the genetic stability of the T-DNA insertion in DP23211 maize (Section V-E, pp. 82-84, and Appendix 4, pp. 335-336, Pioneer 2020). Methods and data from these molecular characterization techniques, provided in Section V and Appendices 2, 3, and 4 of the petition (Pioneer 2020) and reviewed by APHIS, demonstrated that:

- A single, intact T-DNA from PHP74643 was inserted into the genome of DP23211 maize. Results from the SbS analysis identified the presence of two unique junction sites in a representative DP23211 maize individual that was initially identified as PCR positive for the PHP74643 T-DNA insert (Figure 11, p. 66, and Figure 14, pp. 71-72, Pioneer 2020). The presence of these two junction sites was identical across multiple individuals of DP23211 maize that tested positive for the PHP74643 T-DNA insert (Table 7, p. 65 and Appendix 2, pp. 311-332, Pioneer 2020). Additionally, SbS data from control maize and positive control samples supported the results from SbS analysis on DP23211 maize (Section V-B, p. 62, Pioneer 2020).
- The transformation event in DP23211 maize represents a single T-DNA insertion. Results from the SbS analysis demonstrated an absence of unique junction sites within the inserted PHP74643 T-DNA (Section V-B, pp. 60-72, Pioneer 2020),

indicating an absence of molecular rearrangement. Additionally, the SbS analysis demonstrated that no additional insertions or plasmid backbone sequences are present in the genome of DP23211 (Section V-B, p. 63, Pioneer 2020).

- No unexpected plasmid backbone sequences were detected in DP23211 maize.
- The PHP74643 T-DNA insert is stably integrated into DP23211 maize and its progeny. The Southern blot analysis with *DvSSJ1*, *ipd072Aa*, *pmi*, and *mo-pat* gene probes showed that the 5' and 3' genomic borders of the DP23211 insertion are intact and stable across five generations of DP23211 maize during the breeding process. (Tables 8 and 9, p. 74, and Figures 15 and 16, pp. 75-76, and Appendix 3, pp. 333-334, Pioneer 2020).
- The PHP74643 T-DNA insert is functional and segregates according to Mendelian rules of inheritance in DP23211 maize and its progeny. In conjunction with the Southern blot results described above, PCR and herbicide screening of five DP23211 maize breeding generations demonstrated sufficient overlap between expected and observed segregation ratios for each breeding generation of DP23211 maize examined (Table 10, p. 84, Pioneer 2020).

In summary, methods and results provided in Section V and Appendices 2, 3, and 4 of the petition (Pioneer 2020), and reviewed by APHIS, demonstrated that DP23211 maize contains a single, intact PHP74643 T-DNA within its genome. The insertion in DP23211 maize represents a non-complex T-DNA integration event; the data demonstrated no rearrangement of genetic elements in the PHP74643 T-DNA and no truncations in the inserted DNA. Additionally, the PHP74643 T-DNA is stably integrated into the plant genome across multiple breeding generations of DP23211 maize and its progeny.

Expression of inserted DNA, changes in gene expression, new proteins or metabolism

DP23211 maize was developed by Pioneer to exhibit control of CRW pests and tolerance to glufosinate-ammonium herbicides; it also produces the phosphomannose isomerase protein as a selectable marker. These traits are derived from the activity of four gene cassettes within the PHP74643 T-DNA that is integrated into the genome of DP23211 maize. The DvSSJ1 supression cassette and *ipd072Aa* cassette confer control of CRW pests, the *mo-pat* gene cassette confers resistance to glufosinate-ammonium herbicides, and the *pmi* cassette allows growth of plant cells in media with mannose as a carbon source.

DvSSJ1

The DvSSJ1 dsRNA produced in DP23211 maize is targeted to match a portion of the smooth septate junction protein 1 (*dvssj1*) gene sequence from WCR (*Diabrotica virgifera virgifera*) to down-regulate expression of the DvSSJ1 protein, which is primarily expressed in the midgut of WCR, via RNA interference (RNAi) (Pioneer 2020). Expression of DvSSJ1 dsRNA is constitutive in DP23211; the highest concentrations were detected in leaves and young roots, while low amounts were detected in pollen. Reduction in DvSSJ1 protein expression in WCR and subsequent loss of formation of the

gut epithelium barrier and cellular deformities are lethal to these insects (Hu et al. 2019). Efficacy data and mortality of target pest(s) are provided in the petition. Potential impacts to nontarget organisms and the relationship to protein expression are addressed in the respective section below.

Among insecticidal products, RNAi is generally considered more specific than other actives because it is based on nucleic acid sequence in the target pest (Whyard et al. 2009; Bolognesi et al. 2012; Li et al. 2015). MON 87411, the first commercial insecticidal RNAi event, was deregulated by the U.S. Department of Agriculture (USDA) in 2015 and was granted a conditional registration for commercial sale in 2017 by the U.S. Environmental Protection Agency (EPA) (US-EPA 2017). The DvSnf7 suppression cassette contained in MON 87411 produces a 240bp dsRNA that exhibits toxicity to rootworm (Bolognesi et al. 2012; Pan et al. 2016).

ipd072Aa

The *ipd072Aa* gene is derived from *Pseudomonas chlororaphis* (Schellenberger et al. 2016). IPD072Aa is a newly identified insecticidal protein with activity against WCR and other Coleoptera (Boeckman et al. 2019; Carlson et al. 2019). IPD072Aa is a non-pore forming protein that binds to brush border membrane vesicles in the midgut of WCR and disrupts epithelial cells causing breakdown of the epithelial lining (Pioneer 2020). The IPD072Aa protein is expressed constitutively in DP23211; with highest expression observed in roots and whole plants, and lowest expression observed in pollen and grain. Efficacy data and mortality of target pest(s) are provided in the petition. Potential impacts to nontarget organisms and the relationship to protein expression are addressed in the respective section below.

mo-pat

The introduced *mo-pat* gene and its corresponding MO-PAT protein in DP23211 maize is identical to the trait within several crop plants that were previously reviewed by USDA as part of the petition process and are currently in U.S. commercial production (USDA-APHIS 2001, 2005, 2013b, 2019). Thus, the *mo-pat* gene and its corresponding MO-PAT protein in DP23211 maize is already well studied. Maize containing the PAT protein has been commercially grown in the United States since 1996. PAT protein safety has been reviewed and authorized for food and feed use by regulatory authorities in 20 different countries and/or regions. Authorizations for plants developed using genetic engineering that express the PAT protein have been issued in 7 species of plants and total over 450 authorized uses (CERA 2016). The *mo-pat* gene cassette in the PHP74643 T-DNA is responsible for constitutive expression of the MO-PAT protein which confers resistance to phosphinothricin and phosphinothricin-based herbicides.

pmi

The introduced *pmi* gene and its corresponding PMI protein in DP23211 maize is identical to the trait within several crop plants that were previously reviewed by USDA as

part of the petition process and are currently in U.S. commercial production (USDA-APHIS 2021). Thus, the *pmi* gene and its corresponding PMI protein in DP23211 maize is already well studied. The United States EPA has granted an exemption from the requirement of a tolerance for the PMI protein as an inert ingredient in plants (US-EPA 2004). The PMI protein catalyzes the reversible interconversion between mannose-6-phosphate and fructose-6-phosphate. In the presence of PMI, plant cells may survive on media containing mannose as a carbon source, thus allowing PMI to be utilized as a selectable marker (Negrotto et al. 2000; Reed et al. 2001).

Compositional analysis of DP23211 maize

As previously discussed, the integration of the DvSSJ1, *ipd072Aa*, *mo-pat* and *pmi* cassettes in DP23211 maize results in the production of DvSSJ1 dsRNA, IPD072Aa, MO-PAT, and PMI proteins, respectively. To determine if these introduced gene cassettes affected maize metabolism, Pioneer (2020) conducted compositional analyses on DP23211 maize to determine if there were any relevant changes compared to those in the parental line and additional conventional lines as a reference. No significant differences were found in thirty metabolites, nutrients, and antinutrients compared to control and reference corn lines.

In summary, the molecular characterization, protein expression, and compositional analysis of DP23211 maize support a conclusion that there are no unanticipated changes in the engineered plant other than the intended expression of the introduced traits.

D. Potential Plant Pest and Disease Impacts

APHIS assessed whether potential plant pest or disease impacts are likely to result from the transformation process, from DNA sequences from plant pests, or from any other expression products, new enzymes, proteins or changes in plant metabolism or composition in DP23211 maize that are known or anticipated to cause disease symptoms, or to affect plant pests or diseases or plant defense responses. APHIS also assessed whether DP23211 maize is likely to have significantly increased disease and pest susceptibility based on data and observations from field trials on specific pest and disease damage or incidence and any agronomic data that might relate to such damage. Impacts or changes are assessed to determine if they would (1) affect DP23211 and/or result in significant introduction or spread of a damaging pest or disease to other plants; (2) result in the introduction, spread, and/or creation of a new disease; and/or (3) result in a significant exacerbation of a pest or disease for which APHIS has a control program. Any increase in pest or disease susceptibility is evaluated with respect to the context of currently cultivated varieties, the ability to manage the pest or disease, and the potential impact on agriculture.

Plant Protection and Quarantine (PPQ) is an APHIS program that safeguards agriculture and natural resources from the entry, establishment, and spread of animal and plant pests and noxious weeds into the United States of America. PPQ also supports trade and

exports of U.S. agricultural products. PPQ responds to many new introductions of plant pests to eradicate, suppress, or contain them through various programs in cooperation with state departments of agriculture and other government agencies. These may be emergency or longer-term domestic programs that target a specific pest. A variety of insect, plant disease, mollusk, nematode or weed programs exist (USDA APHIS PPQ 2022).

APHIS PPQ has several active management programs for insect pests of maize. These include programs for grasshoppers (Order Orthoptera), old world bollworm (*Helicoverpa armigera*), and Japanese beetle (*Popillia japonica*)(USDA NRCS 2021i; USDA APHIS PPQ 2022). These programs are not expected to be affected by cultivation of DP23211 corn.

Maize itself is not considered a plant pest in the United States and is not expected to/or known to persist in an unmanaged environment (OECD 2003). DP23211 contains noncoding genetic sequences from *Agrobacterium tumefaciens* (border sequences), cauliflower mosaic virus (enhancer and terminators of transcription), and Banana streak virus (acuminata Yunnan strain) (promoter of transcription) (Pioneer 2020). These sequences pose no increase in plant pest risk, in that they are non-coding and used solely to regulate the genes of interest.

Pioneer evaluated the differences between DP23211 maize and its near isoline and/or other conventional maize lines in the incidence of insect predation, plant disease, and other biota at all growth stages (Pioneer 2020). Common arthropod pests observed included aphids (*Aphididae spp.*), fall armyworm (*Spodoptera frugiperda*), corn earworm (*H. zea*), European corn borer (*Ostrinia nubilalis*), flea beetle (*Chaetocnema pulicaria*), Japanese beetles (*Popillia japonica*), spider mites (*Tetranychidae spp.*), corn sap beetle (*Carpophilus spp.*), and thrips (*Frankliniella spp.*). Common microbial pests observed included common rust (*Puccinia sorghi*), maize dwarf mosaic virus, maize rough dwarf mosaic virus, northern corn leaf blight (*Exserohilum turicum*), common smut (*Ustilago maydis*), and gray leaf spot (*Cercospora zeae-maydis*). There were no unexpected differences between DP23211 maize, its near isogenic control, and other reference maize hybrids.

The intended target of the DvSSJ1 dsRNA and IPD072Aa protein in DP23211 maize is control of coleopteran pests in the CRW complex. Pioneer (2020) conducted field efficacy testing and concluded that DP23211 maize was efficacious against CRW. Other than the intended effect on the target pests, the introduced genes did not significantly alter the observed insect pest infestation and disease occurrence or resulting damage on DP23211 over the control or reference lines. As discussed above, there were no significant changes in DP23211 composition that would render DP23211 more susceptible to pests and diseases over its control or reference corn hybrids. The observed agronomic traits also did not reveal any significant changes that would indirectly indicate that DP23211 is or could be relatively more susceptible to pests and diseases over control or reference corn hybrids. Thus, DP23211 is unlikely to be more susceptible to plant pathogens and insect pests than conventional corn. For this reason, DP23211 is unlikely

to differ from conventional corn in its ability to harbor or transmit plant pathogens or pests and cause indirect plant pest effects on other agricultural products.

E. Potential Impacts on Nontarget Organisms Beneficial to Agriculture

DP23211 is engineered for pest resistance and herbicide tolerance. APHIS assessed whether exposure or consumption of DP23211 and the plant incorporated protectants (PIPs) would have a direct or indirect adverse impact on species beneficial to agriculture**.** Organisms evaluated were representatives or surrogates of the species associated with production of the regulated crop in the agricultural environment. The assessment includes an analysis of toxicity and specificity of the PIPs and exposure to sensitive nontarget organisms in the agricultural environment of DP23211 maize. It also may include an analysis of the DP23211 plant compared to the conventional counterpart (or other comparators) with respect to the following: any biologically relevant changes in the phenotype or substances produced which may be novel or expressed at significantly altered amounts that are associated with impacts on organisms beneficial to agriculture, and/or any observations of beneficial organisms associated with the plants.

As with other pesticidal plants, evaluation of DP23211 includes evaluation of the insecticidal compounds themselves with respective modes of action, expression over time and tissue (how much is available for ingestion), taxonomic spectrum of species that are affected by the toxin, and pathways of exposure to the toxin.

DP23211 produces the insecticidal protein named IPD072Aa, insecticidal dsRNA targeting DvSSJ1, the PAT protein for tolerance to glufosinate herbicides, and the PMI protein that was used as a selectable marker during the plant transformation process. PAT and PMI are not known to be toxic to any nontarget organisms beneficial to agriculture. The latter two proteins are present in numerous deregulated crop plants that are commercially available to growers. Therefore, this part of the assessment focuses on the novel insecticidal compounds produced by DP23211.

The IPD072Aa protein expressed by DP23211 was derived from *Pseudomonas chlororaphis* and is part of a family of bacterially derived genes that appear to have insecticidal activity (Schellenberger et al. 2016). The source organism *P. chlororaphis* is a rhizobacterium that promotes plant growth and suppresses some soil-borne plant pathogens. Some strains are commercially available as a plant growth promoter and as a microbial pesticide (Anderson and Kim 2018), and there is a history of safe use for human exposure to the species from which the IPD072Aa protein is derived. Bioinformatic analysis of the IPD072Aa protein revealed no matches to known vertebrate toxins or allergens, and in laboratory assays, the protein degraded in simulated gastric fluid. When diets supplemented with IPD072Aa protein were fed to mice and quail, no negative impacts were observed (Carlson et al. 2019; Pioneer 2020). These pieces of evidence indicate that harm to vertebrates (including humans and mammals) is unlikely.

Lack of harm to vertebrates due to ingestion of the dsRNA of DvSSJ1 produced by DP23211 is supported by the following. First, multiple barriers to uptake of dietary dsRNA exist in mammals (reviewed in (Petrick et al. 2013)). To illustrate, even when mice were fed dsRNA matching the mouse ortholog of an insecticidal RNAi target (vacuolar ATPase), no toxicity or other negative effects were observed (Petrick et al. 2015). Second, the target gene of the insecticidal dsRNA produced by DP23211 is found only in arthropods/invertebrates and bioinformatic analysis yielded no exact 21nt matches outside the genus *Diabrotica* (Pioneer 2020). Finally, in empirical studies, no negative impacts were observed in quail when fed dsRNA of DvSSJ1.

When rats and broiler chickens were fed corn grain from DP23211, which expresses both the IPD072Aa protein and dsRNA of DvSSJ1, no adverse effects were observed (Smith et al. 2021). In summary, the information provided suggests no adverse impacts to humans and vertebrate non-target organisms due to the insecticidal compounds produced by DP23211; therefore, subsequent analysis below is focused on invertebrate non-target organisms.

As described above, the IPD072Aa protein is derived from *P. chlororaphis*, strains of which may be used to promote plant growth and for control of microbial pathogens and various insect and nematode pests (Anderson and Kim 2018). The isolated protein was nevertheless presumed to be specific to Coleoptera, and emphasis was placed on beetles in the risk assessment described in the petition.

IPD072Aa is 86 amino acids in length (Schellenberger et al. 2016) and is a non-pore-forming protein that binds to the WCR midgut. The submitted petition states that the IPD072Aa protein forms a dimer in solution and in the plant, and that it dissociates after WCR ingest it. In assays with larvae fed on artificial diet with IPD072Aa, presence of the protein was observed throughout the lumen of the gut and resulted in significant damage to enterocytes.

Microbially-produced protein was used in bioassays with nontarget arthropods and to study the mode of action in sensitive target pest(s). Methods that, in combination, are commonly used to demonstrate equivalence of microbially and plant produced proteins are summarized in (Raybould et al. 2013). These methods for protein characterization should be supplemented with bioassays using a sensitive insect species in order to demonstrate functional equivalence.

Equivalency between microbially-produced protein and DP23211-produced protein was claimed using protein run on separate gels for SDS-PAGE and Western blot (Pioneer 2020). In total, the developer was able to match 77% of the DP23211 plant-derived protein to the microbially-derived protein used in biosafety testing (Pioneer 2020). Equivalent bioactivity of pesticidal proteins can also be inferred by using an assay with a sensitive insect, but these tests were conducted non-concurrently (Pioneer 2020).

The other insecticidal compound produced by DP23211 is dsRNA that targets DvSSJ1. Smooth septate junctions are present in arthropods and some other invertebrates

contribute to the integrity of the midgut barrier. There are two orthologs present in *Drosophila melanogaster*, both of which contribute to the integrity of the midgut barrier. DvSSJ1 is an ortholog of snakeskin; (dvssj2 is ortholog of mesh), (Hu et al. 2016). Knockdown of ssk expression in larvae and adults of *Drosophila melanogaster* results in increased permeability of the midgut (Yanagihashi et al. 2012; Salazar and Yamamoto 2018; Izumi et al. 2019). DvSSJ1 is expressed primarily in the midgut of WCR, with lower levels of expression in other tissues (Hu et al. 2019).

Insecticidal RNAi functions whereby long dsRNA ingested by the insect enters the cells in the midgut where it is then cleaved into smaller pieces called short interfering RNAs (siRNA) (Price and Gatehouse 2008). These siRNAs bind to complementary mRNAs and results in knockdown of the target gene, chosen because it is essential to the insect pest (Baum et al. 2007). Though these siRNAs are ultimately responsible for silencing expression of essential genes, longer sequences of at least 60bp in length must be fed to susceptible WCR to exert insecticidal effect (Bolognesi et al. 2012; Li et al. 2015; Hu et al. 2020).

The dvSSJ1 transcript produced by DP23211 is expressed as an inverted repeat of a 210 nt fragment (Pioneer 2020) with an intervening intron for hairpin formation. After ingestion by WCR, subsequent cleavage results in siRNAs with 21nt as the most abundant size (Hu et al. 2019). DvSSJ1 dsRNA in diet bioassays acts in a dose-dependent manner against susceptible insects (Hu et al. 2019).

Some methods that, in combination, demonstrate equivalence of *in vitro* dsRNA to dsRNA produced *in planta* are described by (Urquhart et al. 2015), but these types of studies were not done for dsRNA of DP23211. Activity against WCR was implied to be a positive control, but these studies were done non-concurrently with other bioassays. Bioassays were conducted with RNase-free water as a negative control, rather than dsRNA of an exogenous target gene, *e.g.* see (Whyard et al. 2009; Bolognesi et al. 2012; Li et al. 2015; Pan et al. 2016; Haller et al. 2019; Hu et al. 2019; Pan et al. 2020; Vélez et al. 2020), and positive controls involved use of an inorganic compound with insecticidal activity, rather than dsRNA targeting an endogenous gene, *e.g.* (Haller et al. 2019).

Because *dvssj1* inhibits expression of a membrane junction protein in the insect (mid)gut, and the IPD072Aa protein is small compared to proteins in other commercially available PIPs, the possibility of interaction was investigated. Data provided in the petition suggests some small synergistic effect between the two toxins but the developer concluded that such results were not biologically relevant (Pioneer 2020).

Assessment of risk to nontarget organisms is informed by expression in the plant and exposure pathways. Expression of IPD072Aa and dsRNA of *dvssj1* in DP23211 was reported from different tissues in corn plants collected from six sites in a single field season. Due to limited replication, the highest recorded concentration for each relevant plant tissue was used in this assessment. Additionally, fresh weight conversions were not calculated, so dry weight values were used. Because dry weight concentrations will be

higher than fresh weight conversions, this adds support to conclusions from a limited study.

Though variability in expression is to be expected, some trends can be observed. Expression of the IPD072Aa protein was highest in roots (highest amount recorded was 84 ng/mg dry weight), followed by leaf and other aboveground vegetative parts of the plant (highest sample at 39 ng/mg dry weight). Protein concentration was an order of magnitude lower in grain (4.8 max) and pollen at 1.3 ng/mg dry weight.

Though protein concentration is best quantified using lyophilized material as a starting point, nucleic acids are quantified using fresh or flash-frozen tissue. Fresh weight and converted values in dry weight are reported in the petition for *dvssj*. Using fresh weight values, concentration of *dvssj1* is highest in the leaf, followed by root. Amount of *dvssj1* in the pollen is much lower at 0.9 pg. When converted to dry weight, both root and leaf have similar concentration, with max values of 94 and 113 pg, respectively. Pollen amounts are similarly significantly lower at 2 pg.

Exposure of nontarget organisms to IPD072Aa and *dvssj1* in DP23211 can occur directly or indirectly. Direct exposure could occur, for example, by a pollinator feeding on pollen, a scavenger or detritivore feeding directly on the plant or sloughed-off material, or predators feeding on pollen or vegetative plant parts. Natural enemies that feed on plants may do so to obtain moisture, to sustain themselves in periods of prey scarcity, or to supplement their diet even when sufficient prey are present (Lundgren 2009). Indirect exposure is generally understood to constitute trophic exposure, whereby a natural enemy is exposed to the plant-produced toxin via a prey item. Use of prey species that are not susceptible to the toxin excludes any confounding effects of intoxicated or suboptimal prey. Finally, any community-level effects due to a plant-incorporated toxin would not be a direct result of exposure to any toxin, but could be considered an indirect effect of a plant-produced toxin through reduced prey abundance.

The IPD072Aa protein and DvSSJ1 dsRNA were tested for toxicity with a range of invertebrate species, especially insects. Species were selected based on amenability to lab testing, to encompass a variety of species across the taxonomic spectrum, and to include species representing different ecological niches. Amounts of protein or dsRNA, respectively, used in bioassays were either similar to or greater than what was quantified in the plant, in order to support a conclusion that these groups would not be negatively impacted by protein exposure expected in the environment. Mortality to target pests, such as WCR and other *Diabrotica* spp., was evidenced through diet bioassays and/or efficacy tests on DP23211 plants. Emphasis was placed on predatory Coleoptera because of their relatedness to the target pests and importance as natural enemies in agroecosystems.

For taxonomic specificity of the IPD072Aa protein, western corn rootworm was unsurprisingly the most sensitive insect tested, with an approximate LC50 of 26 ng/mg diet. Effects on southern corn rootworm appeared to be primarily sublethal. Another Chrysomelid (but that is not a corn pest), Colorado potato beetle, did not exhibit any mortality or sublethal effects from the IPD072Aa protein. Regarding Coccinellidae,

another large family of beetles that is related to Chrysomelidae, some mortality was observed with higher doses (500ng/mg) of the protein in 2 out of the 4 species tested. At a lower dose of 100 ng/mg diet, sublethal effects were also observed in 2 out of the 4 species. For tenebrionid beetles, some lower level of sensitivity was observed. Finally, the 4 species of Lepidoptera that were tested did not exhibit any sensitivity to the IPD072Aa protein.

For a collembolan species, some reproductive effects were observed at 500ng dosage. For a parasitoid wasp, some low level mortality was observed in a non-dose-dependent manner. Green lacewing (Neuroptera) did not experience mortality effects or effects on successful pupation (other sublethal effects were not reported).

Based on the analysis of the information provided in the petition on the safety and expression level of the DvSSJ1 dsRNA and IPD072Aa protein, some of which is present in peer-reviewed literature, APHIS concludes that exposure to and/or consumption of DP23211 maize is unlikely to have any adverse impacts to organisms beneficial to agriculture.

F. Potential for Enhanced Weediness of DP23211 Maize

APHIS assessed whether DP23211 maize is likely to become more weedy (i.e., more prevalent, competitive, damaging or difficult-to-control in situations where it is not wanted) than the unmodified progenitor from which it was derived or other varieties of the crop currently under cultivation. This assessment considers the basic biology of maize, the situations in which maize volunteers are considered weeds, and an evaluation of DP23211 maize compared to its near isogenic control and other reference maize hybrids. Evaluations on DP23211 maize centered on characteristics related to establishment, competitiveness, reproduction, survival, persistence and/or spread that could influence weediness and the ability to manage maize as a volunteer; these evaluations were undertaken in laboratory and field studies. Laboratory studies primarily focused on seed viability and germination/dormancy, while field studies focused on maize characteristics, including early stand count, days to flowering, height, lodging, final stand count, days to maturity, pollen viability, kernel rows per ear, kernels per ear, kernels per row, harvest grain moisture, yield, 100-kernel weight, and seed germination and dormancy (Pioneer 2020). Additionally, responses to various abiotic stresses (e.g., above average rainfall, wind, hail, etc.) were observed and evaluated during the confined field testing of DP23211 maize (Pioneer 2020).

In the United States, maize is not listed as a weed in the major weed references (Crockett 1977; Holm et al. 1979; Holm et al. 1997) and it is not designated as a noxious weed by the federal government (USDA-APHIS 2020). Maize is unable to establish outside agriculture, as evidenced by the lack of reports of such behavior despite being one of the most widely cultivated grains in the world, and by data from controlled experiments where maize plantings left unharvested resulted in no feral plants within a year or two after planting (Raybould et al. 2012; Sammons et al. 2014). However, maize has been mentioned as an agricultural weed (i.e., volunteer plants) (Marquardt et al. 2013). Maize

does not possess any of the attributes commonly associated with weeds such as long persistence of seed in the soil, the ability to disperse, invade, and become a dominant species in new or diverse landscapes, or the ability to compete well with native vegetation (Baker 1965). Maize seeds are retained on the cob covered in a husk and are poorly dispersed, have no innate dormancy and are susceptible to low temperatures, although some seeds may overwinter and germinate when weather conditions allow; however germinating seedlings and plants are sensitive to cold and do not survive freezing winter conditions (Hoeft et al. 2000; OECD 2003; OGTR 2008; Andersson and de Vincente 2010). Although maize seed does not shatter, kernels are often scattered by harvest equipment or foraging wildlife, and some may survive to create volunteer plants the following year. Similar to conventional maize volunteers, herbicide resistant maize volunteers, including DP23211 maize volunteers, can be managed by optimizing mechanical cultivation, crop rotation, and the careful selection of the modes of action for pre-emergent and post-emergent herbicides to balance competing herbicide sensitivities between volunteers and the rotational crop (Vencill et al. 2012).

In order to evaluate germination and dormancy in DP23211 maize, Pioneer performed laboratory studies utilizing warm, cold, and diurnal conditions. Across these three conditions, DP23211 maize seeds demonstrated a germination rate no lower than 99%; this result was comparable to the germination rate of its near isogenic control and other reference maize hybrids under the same conditions. While these data demonstrate similar germination rates between DP23211 maize, its near isogenic control, and other reference maize hybrids, these data also suggest that seed dormancy rates between the DP23211 and its comparators are likely similar. This conclusion is reinforced by the absence of hard or fresh seed among the DP23211 maize seeds that did not germinate; the presence of these two characteristics is generally associated with seed dormancy germinate (Anderson 1996; Pioneer 2020).

Pioneer performed field evaluation of DP23211 maize across 12 sites spanning regions of the United States where DP23211 maize is intended to be (Pioneer 2020). The collected data demonstrated no substantial differences between DP23211 maize and its comparators for early stand count, days to flowering, height, lodging, days to maturity, pollen viability, kernel rows per ear, kernels per ear, kernels per row, harvest grain moisture, and 100-kernel weight. Statistically significant differences were identified in days to flowering and final population, however these differences are not anticipated to be biologically meaningful as they are within the reference range for each measurement. The values for other agronomic traits were observed to be within the reference range of other corn hybrids, suggesting that variation is within the norm of commonly cultivated maize varieties. These data provide evidence that DP23211 maize grows and develops in a similar manner to its conventional maize comparators.

Additionally, there were no substantial differences in abiotic stress responses between DP23211 maize and its near isogenic control and other reference maize hybrids across the 12 sites during the 2018 growing season (Pioneer 2020). These data suggest that DP23211 maize responds to abiotic stressors in a similar manner as its conventional maize comparators, and is further reinforced by an absence of unexpected abiotic stress

responses during the years that DP23211 maize was field tested within the United States. Also, as previously examined, DP23211 maize did not exhibit any unexpected responses to naturally occurring insects (other than CRW) or diseases compared to its near isogenic control or reference maize varieties.

The data show that neither the corn rootworm resistance traits nor the marker traits altered the weediness potential of DP23211 maize compared to the conventional control based on the assessed phenotypic and agronomic traits. This conclusion, in conjunction with the cultivation of existing insect resistant maize varieties in the United States, indicates that DP23211 maize is unlikely to be any more difficult to control as a volunteer in subsequent seasons after its planting. There are numerous methods to effectively manage volunteer maize in agricultural fields (Jeschke and Doerge 2010); given that absence of increased weediness potential in DP23211 maize, existing methods that are effective in control currently maize volunteers, are also likely to be effective in controlling DP23211 maize volunteers.

In summary, DP23211 maize is unlikely to persist as a troublesome weed or to have an impact on current weed/volunteer management practices, based on the agronomic laboratory data, field data, and literature survey concerning weediness potential of the crop. These data suggest that DP23211 maize is no more likely to become a weed than conventional varieties of maize.

G. Potential Impacts on the Weediness of Any Other Plants with which DP23211 maize Can Interbreed

Gene flow is a natural biological process with significant evolutionary importance. A number of angiosperm taxa are believed to be derived from hybridization or introgression between closely related taxa (Grant 1981; Rieseberg and Wendel 1993; Soltis and Soltis 1993; Hegde et al. 2006), and even in the existing floras, the occurrence of hybridization or introgression is reported to be widespread (Stace 1987; Rieseberg and Wendel 1993; Peterson et al. 2002). It has been a common practice by plant breeders to artificially introgress traits from wild relatives into crop plants to develop new cultivars (Khoury et al. 2013). However, gene flow from crops to wild relatives is also thought of as having a potential to enhance the weediness of wild relatives, as observed in rice, sorghum, sunflower, and a few other crops (see Table 1 in (Ellstrand et al. 1999). This topic is covered in two sections: 1) the potential for gene flow, hybridization and introgression from the DP23211 maize to sexually compatible relatives, including wild, weedy, feral or cultivated species in the United States and its territories, and 2) if gene flow is likely, then risk potential with respect to weediness of those taxa based on the phenotypic changes that have been observed in DP23211 maize.

Potential for gene flow, hybridization and gene introgression

Cultivated maize, *Z. mays* subsp. *mays*, is a member of the grass family Poaceae. The genus *Zea* has five species: *Z. mays*, *Z. diploperennis*, *Z. luxurians*, *Z. nicaraguensis*, and

Z. perennis. Z. mays is further divided into four subspecies: *mays*, *huehuetenangensis*, *mexicana* and *parviglumis*. *Z. mays* subsp. *mays* is the only cultivated species of the genus *Zea*; the other species and subspecies are referred to as teosintes (OGTR 2008). Teosinte is a common name applied to several distinct wild, annual and perennial diploid and tetraploid taxa native to a region extending from Northern Mexico to Western Nicaragua and normally confined to the tropical and subtropical regions of Mexico, Guatemala, and Nicaragua (OGTR 2008; Andersson and de Vincente 2010).

Except for *Z. perennis*, teosintes can be crossed with cultivated maize to produce fertile hybrids (Doebley 1990; OGTR 2008). However, there are barriers that reduce or prevent gene flow between maize and teosinte in the environment. For example, temporal and spatial factors isolate *Z. mays* subsp. *parviglumis* from maize, and there is some genetic incompatibility between maize and *Z. luxurians* and *Z. mays* subsp *mexicana*. Experimental and molecular data suggests that maize and teosintes can hybridize when grown in close proximity, and hybridization occurs sporadically and at very low rates (Doebley 1990; Baltazar et al. 2005). On the other hand, *Z. mays* subsp. *parviglumis* and maize can hybridize readily at higher rates (Ellstrand et al. 2007). Several features of teosinte inflorescences and pollen and the existence of incompatibility systems in teosintes may discourage pollination of teosintes by other taxa (Baltazar et al. 2005). Introgression between maize and teosintes is also limited by the geographical distribution of teosintes, which have natural ranges limited to Mexico and certain parts of Central America.

A search of the Plants Database yielded results showing that *Z. mexicana* (Syn. *Z. mays* subsp *mexicana*) is listed as present in Florida, Alabama and Maryland, having been introduced from Mexico (USDA NRCS 2021h); *Z. perennis* is listed in Texas and South Carolina (USDA NRCS 2021g). *Z. diploperennis* and *Z. luxurians* are also listed, but there is no information about their location and status (USDA NRCS 2021f, e). Experts familiar with the teosinte collections in the United States have been previously consulted and are not aware of the presence of any naturalized or native populations of teosintes in the United States (USDA-APHIS 2013a). Therefore, introgression of DP23211 maize into teosinte is unlikely in the United States.

The genus most closely related to *Zea* is *Tripsacum*, a genus with 16 species. Plants in this genus are rhizomatous perennial grasses with geographical distribution extending from the northern United States to Paraguay in South America. Some species are present as cultivated or wild species in the continental United States, including *Tripsacum dactyloides*, *T. floridatum* and *T. laceolatum* (USDA NRCS 2021d, c, a); *T. fasciculatum* and *T. latifolium* occur in Puerto Rico (USDA NRCS 2021b, j). *Tripsacum* species (2n=18) can be represented by diploid, triploid, tetraploid and higher ploidy levels. All species with the same ploidy levels can be crossed with *Zea* species (2n=20) under experimental lab conditions with difficulty and the hybrid offspring are sterile (Galinat 1988; OGTR 2008; Andersson and de Vincente 2010).

Maize is a predominantly wind pollinated, outcrossing plant species. Insect pollination has not been reported. Maize cultivars and landraces are diploid plants (2n=20) that can

crossbreed to a large degree. However, some evidence for genetic incompatibility exists within the species (e.g., popcorn x dent and Mexican maize landraces x Chalco teosinte crosses; see (Wozniak 2002). There is a difference in floral synchrony between male (tassel) and female (silk) flowers on the same plant; the tassels begin shedding pollen before female flowers are receptive to fertilization. Typically tassels shed pollen for 2-14 days depending on environmental conditions. Because female flower development lags behind that of tassel and anthers with minimum overlap, the rate of self-pollination is only approximately 5% (Sleper and Poehlman 2006). Pollen viability has been variously described as lasting from 10-30 minutes (Coe et al. 1988) to up to 2 hours (Luna et al. 2001). Due to weight and diameter, most pollen grains are deposited within 60 feet of the source plant. Cross pollination between a donor field and receptor field can occur over a 7 day period (Coe et al. 1988; OGTR 2008). However, adverse consequences of gene flow from DP23211 maize to wild or weedy related species in the United States are highly unlikely.

Gene flow potential of DP23211 maize was evaluated thoroughly. The introduced DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI proteins in DP23211 maize are not expected to change the ability of the plant to interbreed with other plant species. Furthermore, the APHIS evaluation of data provided by Pioneer of agronomic and phenotypic properties of DP23211 maize, including those characteristics associated with reproductive biology such as seed germination and dormancy, early stand count, plant height, final stand count, grain moisture, test weight, yield and pollen morphology and viability indicated no unintended changes likely to affect the potential for gene flow from DP23211 maize to sexually compatible species (Pioneer 2020). The potential for gene flow to occur specifically between insect-resistant crop varieties and their sexually compatible relatives has been previously addressed (Mallory-Smith and Sanchez Olguin 2011). Therefore, the potential for gene flow and introgression of the insect resistant traits from DP23211 maize to other maize hybrids and its consequences are anticipated to be similar to those as for existing commercial maize hybrids.

Many conditions have been identified that are required for gene flow and introgression to occur between a crop and its wild relatives (Carpenter et al. 2002; Jenczewski et al. 2003; Stewart et al. 2003; Owen 2005), including flowering synchrony, abundance and method of pollen spread, distance of pollen movement, genetic compatibility, and environmental conditions pertinent to cross-pollination, but the foremost condition is the presence of wild relatives within pollen or seed dispersal range from the crop. In the United States, the lack of sexually compatible wild relatives of *Z. mays* ssp. *mays* precludes the opportunity for gene flow to occur between cultivated maize and its wild relatives. Based on the information presented in the petition and in relevant literature, APHIS has reached the following conclusions: The genetic modification in DP23211 maize is not expected to increase the potential for gene flow, hybridization and/or introgression to sexually-compatible taxa compared to the non-transgenic recipient or other hybrids of the crop commonly grown. Gene flow, hybridization and/or introgression of genes from DP23211 to other sexually-compatible relatives with which it can interbreed is not likely to occur in the United States and its territories.

Potential for enhanced weediness of recipients after hybridization and/or introgression

Based on the data presented in the petition, DP23211 maize does not exhibit characteristics that may cause it to be any weedier than other cultivated maize based on the data presented in the petition (Pioneer 2020). Furthermore, none of the sexually compatible-relatives of maize in the United States are considered to be weeds in the United States (Holm et al. 1979). Therefore, even in the extremely unlikely event of successful hybrids and/or introgression between DP23211 maize and its wild relatives, the inserted transgenes of DP23211 maize are unlikely to transform its wild relatives into more weedy species. Moreover, its potential impact due to the extremely limited potential for gene introgression into teosinte and *Tripsacum* species is not expected to be any different than that of other cultivated maize varieties. Based on the above considerations, DP23211 maize is unlikely to adversely impact sexually-compatible wild relatives or their weediness characters.

Based on the information presented in the petition and in relevant literature, APHIS has reached the following conclusions. The genetic modification in DP23211 maize is not expected to increase the potential for gene flow, hybridization, and/or introgression to occur to sexually-compatible taxa compared to the non-transgenic recipient or other varieties of maize that are commonly grown. Gene flow, hybridization, and/or introgression of genes from DP23211 maize to other sexually-compatible relatives, including wild, weedy, feral or cultivated species in the United States and its territories is not likely to occur. Furthermore, both the maize and its sexually compatible relative species are not considered weedy or invasive, and the phytase expression conferred by genetic engineering is not likely to increase the weediness of these species. The modified phenotype is not expected to affect the current ability to control these species in situations where they are considered weedy or invasive; the following measures are still available for their control: herbicides, tillage and other methods. Therefore, DP23211 maize is not expected to increase the weed risk potential of other species with which it can interbreed in the United States and its territories.

H. Potential Changes to Agriculture or Cultivation Practices

APHIS assessed whether significant changes to agricultural or cultivation practices from adoption of the DP23211 maize are likely to impact plant diseases or pests or their management, including any APHIS control programs. This includes consideration of any changes in pesticide applications, tillage, irrigation, harvesting, etc. as they relate to plant pests and diseases.

DP23211 produces two insecticidal compounds for control of rootworm beetles, the PAT protein for tolerance to glufosinate, and the PMI protein that had been used as a selectable marker during product development. Due to the function of the introduced genetic material in DP23211, the only anticipated change to agricultural and cultivation practices due to its adoption would be regarding insecticide applications.

There is already widespread adoption of corn hybrids with herbicide tolerance and Coleoptera resistance across the US corn belt where corn rootworms are present (Duke 2014; USDA NASS 2020). The gene encoding PMI and associated regulatory sequences introduced into DP23211 maize have been granted an exemption for the requirement of a tolerance in all plants by EPA (US-EPA 2004). Additionally, the phosphomannose isomerase (PMI) protein used as a selectable marker is already present in a variety of deregulated events (USDA-APHIS 2022); its function in antibiotic resistance for use as a selectable marker during plant transformation has no impact on agricultural practices.

The phosphinothricin acetyltransferase (PAT) protein for tolerance to glufosinate-ammonium herbicides is already present in a number of commercial corn hybrids (USDA-APHIS 2022). Use of glufosinate ammonium herbicides is common in areas with corn cultivation (Duke 2014; USDA NASS 2020). The gene encoding PAT and associated regulatory sequences introduced into DP23211 maize has been granted an exemption for the requirement of a tolerance in all plants by EPA (US-EPA 1997). Additionally, the phosphinothricin acetyltransferase (PAT) protein is already present in a variety of deregulated events (USDA-APHIS 2022).

Overall, cultivation of Bt crops has resulted in a general decrease in insecticide sprays. However, it should be noted that insecticidal sprays for CRW pests are no longer in widespread use, and furthermore, DP23211 does not eliminate the need for insecticides to control insect pests of corn. Insecticidal seed treatments are almost always used for control of soil-dwelling pests and above-ground pests that are targeted by the systemic insecticide. On the other hand, two scenarios could lead to an increase in insecticide usage (or, reversion back to high rates that were used in the past). One possibility would be if adoption of DP23211 causes resurgence of any secondary pests due to reduced efficacy of the natural enemy community if predatory arthropods are impacted by DP23211. Decreased predator abundance and/or function could result in more frequent insecticide applications to control these secondary outbreaks. The other possibility would be if WCR were to evolve resistance to DP23211. If resistant WCR were to evolve and spread, there could be another return to in-furrow insecticide sprays at planting or aerial sprays during adult emergence, as has already occurred for other corn events targeting this pest (Dunbar et al. 2016).

APHIS could not identify any significant changes to agricultural or cultivation practices (e.g. pesticide applications, tillage, irrigation, harvesting, etc.) from adoption of DP23211 maize; therefore, no impact on plant diseases or pests, or their management is likely to occur.

I. Potential Impacts from Transfer of Genetic Information to Organisms with which DP23211 Maize Cannot Interbreed

APHIS examined the potential for the new genetic material inserted into DP23211 maize to be horizontally transferred without sexual reproduction to other organisms and whether such an event could lead directly or indirectly to disease, damage, injury or harm to

plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

The horizontal gene transfer (HGT) between unrelated organisms is one of the most intensively studied fields in the biosciences since 1940, and the issue gained extra attention with the release of transgenic plants into the environment (Dröge et al. 1998). Potential risks from stable HGT from organisms developed using genetic engineering to another organism without reproduction or human intervention have been reviewed (Keese 2008). Mechanisms of HGT include conjugation, transformation and transduction, and other diverse mechanisms of DNA and RNA uptake and recombination and rearrangement, most notably through viruses and mobile genetic elements. HGT has been a major contributor to the spread of antibiotic resistance amongst pathogenic bacteria; emergence of increased virulence in bacteria, eukaryotes and viruses; and, over extended time scales, to major transitions in evolution (Brown 2003; Keeling and Palmer 2008; Keese 2008).

Potential for horizontal gene transfer to bacteria, fungi, or invertebrates

DP23211 maize contains protein coding regions derived from *E. coli* (*pmi*), *P. chlororaphis* (*ipd072Aa*), and *S. viridochromogenes* (*mo-pat*); it also contains small, non-coding regions from *A. tumefaciens* (e.g., sequences related to the T-DNA), *Saccharomyces cerevisiae* (i.e., flippase recombination sites), and bacteriophage lambda (i.e., loxP and AttB recombination sites; Pioneer 2020).

HGT and expression of DNA from a plant species to bacterial, fungal or invertebrate species is unlikely to occur based on the following observations. Although there are many opportunities for plants to directly interact with fungi and bacteria (e.g., as commensals, symbionts, parasites, pathogens, decomposers, or in the guts of herbivores) and with invertebrates as plant pests, there are almost no evolutionary examples of HGT from eukaryotes to bacteria or from plants to fungi or invertebrates (Keese 2008). Examples of HGT between eukaryotes and fungi primarily involve gene acquisition or transfer by fungi to or from other distantly related fungi or bacteria (Keeling and Palmer 2008; Keese 2008) and HGT between plants and fungi is extremely rare (Richards et al. 2009). Examples of HGT between plants and invertebrates are also extremely rare, and most examples of HGT in insects involve acquisition of genes from their pathogens or endosymbionts (Keese 2008; Zhu et al. 2011; Acuna et al. 2012).

HGT from and expression in bacteria of the foreign DNA inserted into the nuclear genome of the genetically modified plant is unlikely to occur. First, many genomes (or parts thereof) have been sequenced from bacteria that are closely associated with plants including *Agrobacterium* and *Rhizobium* (Wood et al. 2001; Kaneko et al. 2002). There is no evidence that these organisms contain genes derived from plants. HGT from plants to bacteria is a very low frequency event, primarily because functional and selective barriers to HGT increase with genetic distance (Keese 2008). Second, in cases where review of sequence data implied that HGT occurred, these events are inferred to occur on an evolutionary time scale on the order of millions of years (Koonin et al. 2001; Brown 2003; EFSA 2009). Third, transgene DNA promoters and coding sequences are

optimized for plant expression, not prokaryotic bacterial expression. Thus, even if HGT occurred, proteins corresponding to the transgenes are not likely to be produced. Fourth, the European Food Safety Authority (EFSA 2009) has evaluated HGT from the use of antibiotic resistance marker genes and concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of humans or animals, or in the environment, is very rare or remote.

Potential for horizontal gene transfer to viruses

APHIS also considered whether horizontal transfer of DNA from DP23211 to plant viruses was likely to occur and would lead to the creation or selection of plant viruses that are more virulent or have a broader host range. DP23211 maize contains small, non-coding regions from Cauliflower Mosaic Virus (35S enhancer and terminator regions), and Banana Streak Virus (acuminata Yunnan strain) (promoter region; Pioneer 2020). This issue has been considered before by other science review panels and government regulatory bodies (EPA-FIFRA-SAP 2006; Keese 2008). HGT is not unusual among plant viruses; however, this is generally limited to exchange between viruses present in the same host organism in mixed infections and most commonly involves homologous recombination, relying on sequence similarity at the point of crossover (Keese 2008). HGT from virus sequences engineered into plants has been demonstrated with infecting or challenge viruses, including DNA viruses (e.g., gemini viruses that replicate in the nucleus; see (Frischmuth and Stanley 1998) and RNA viruses (which typically replicate in the cytoplasm); however most have been under conditions that favor recombination to restore a defective virus (Fuchs and Gonsalves 2007; Keese 2008; Thompson and Tepfer 2010). Populations of recombinants between virus transgenes expressed in transgenic plants infected with related viruses are similar to recombinants found in mixed infections of the same viruses in non-transgenic plants, indicating that there was no novel recombination mechanism in the transgenic plants and no increased risk is expected over what is expected from mixed infections (Keese 2008; Turturo et al. 2008). Non-homologous recombination in HGT among viruses or between virus transgenes and infecting viruses can occur, but frequently results in gene deletions which can result in nonviable viruses (Morroni et al. 2013). Depending on the particular virus and sequences involved, various hot-spots for recombination have been found in both coding and noncoding regions and strategies in the design of transgenes to avoid recombination have been suggested. No recombinant or undesirable viruses with new properties have been detected for over at least 8-10 years in field tests or during commercial growth of virus-resistant plum, squash, or papaya engineered with genes from viruses that have been deregulated in the United States (Fuchs and Gonsalves 2007).

Potential for horizontal gene transfer to parasitic plants

Evidence for HGT from plants to other plants is limited to two specific scenarios: (1) exchange of genes between a parasitic plant and its host; and (2) exchange of genes between cells of two plants living in close proximity, such as in a graft junction. In both cases, this type of HGT requires physical contacts between the two plants. Most cases of HGT in plants involve transfer of mitochondrial genomes, which are primarily maternally

inherited in plants to other mitochondria genomes (Barr et al. 2005), and mostly involve parasitic plants and their hosts (Richardson and Palmer 2007). Recently, a comparative genomics analysis implicated HGT for the incorporation of a specific genetic sequence in the parasitic plant, *Striga hermonthica* (purple witchweed) from its monocot host (Yoshida et al. 2010). According to this study, the incorporation of the specific genetic sequence (with an unknown function) occurred between sorghum and *S. hermonthica*. However, this HGT occurred before speciation of *S. hermonthica* and related *S. gesnerioides* (cowpea witchweed) from their common ancestor. Furthermore, *S. hermonthica* is not found in the United States and *S. asiatica*, another related parasite of cereal crops, is only present in North Carolina and South Carolina (USDA NRCS 2021i). More recent studies demonstrated that in a few parasitic species of the Rafflesiaceae family, out of several genetic sequences examined, about 2.1% of nuclear (Xi et al. 2012) and 24-41% of mitochondrial (Xi et al. 2013) gene transcripts appeared to be acquired from their obligate host species. However, all the above-mentioned instances of HGT between parasitic plants and their hosts were reported to be of ancient origins, on an evolutionary time scale spanning thousands to millions of years ago. Furthermore, in DP23211 maize crop, the DNA sequences were inserted into the nuclear genome, not the mitochondrial genome (Pioneer 2020).

If DP23211 maize becomes infected by a parasitic plant or is naturally grafted to another plant, there is a very low probability that HGT could result in the other plant acquiring DNA from it. However, in both scenarios, this newly introduced DNA would likely reside in somatic cells; with little chance of reaching the germ cells, this introduced DNA could not persist in subsequent generations unless the recipient plant reproduced asexually from the affected cells.

Based on the above analysis, APHIS therefore concludes that HGT of the new genetic material inserted into DP23211 maize to other organisms is highly unlikely, and is not expected to lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

J. Conclusion

APHIS has reviewed the information submitted in the petition, supporting documents, public comments in response to Federal Register notices concerning this petition, and other relevant information to assess the plant pest risk of the DP23211 maize compared to its near isogenic comparator and other maize reference hybrids. APHIS concludes that the DP23211 maize is unlikely to pose a plant pest risk based on the following findings:

- No plant pest risk was identified from the transformation process or the presence of new genetic material in DP23211 maize because the *Agrobacterium tumefaciens* transformation vector was disarmed, , and the plant pest sequences inserted do not cause disease or create an infectious agent.
- No increase in plant pest risk was identified in DP23211 due to unexpected and unrelated effects from expression of the inserted genetic material for control of the target pests.

- Disease and pest incidence and/or damage were not observed to be significantly increased or atypical in DP23211 maize compared to its near isogenic comparator and other maize reference hybrids during field trials and targeted studies conducted in growing regions representative of where DP23211 maize is expected to be grown. Observed agronomic traits also did not reveal any significant differences that would indirectly indicate DP23211 maize is more susceptible to pests or diseases**.** Therefore, no plant pest effects are expected on these or other agricultural products and no impacts are expected to APHIS pest control programs.
- Exposure to and/or consumption of DP23211 maize is unlikely to have any adverse impacts on organisms beneficial to agriculture based on APHIS' analysis of studies on DP23211 maize food and feed safety and composition.
- DP23211 maize is no more likely to become a weed or become weedier than conventional varieties of the crop based on its observed agronomic characteristics, weediness potential of the crop, and current management practices available to control DP23211 maize as a volunteer.
- DP23211 maize is not expected to increase the weed risk potential of other species with which it can interbreed in the United States or its territories. Gene flow, hybridization, and/or introgression of inserted genes from DP23211 maize to other sexually compatible relatives with which it can interbreed is not likely to occur. Any possible introgression into teosintes or *Tripsacum* species of the new phenotype conferred by genetic modification is not likely to increase the weediness of these relatives or affect the current ability to control them in situations where they might be considered weedy or invasive.
- Significant changes to agricultural or cultivation practices (e.g., pesticide applications, tillage, irrigation, harvesting, etc.) from adoption of DP23211 maize were not identified and are not likely to increase plant diseases or pests or compromise their management.
- Horizontal gene transfer of the new genetic material inserted into DP23211 maize to other organisms is highly unlikely and is not expected to lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

K. References

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