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SCIENTIFIC OPINION

Assessment of genetically modified maize DP910521 (application GMFF-2021-2473)

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Abstract

Genetically modified (GM) maize DP910521 was developed to confer resistance against certain lepidopteran insect pests as well as tolerance to glufosinate herbicide; these properties were achieved by introducing the *mo-pat, pmi and cry1B.34* expression cassettes. The molecular characterisation data and bioinformatic analyses did not identify issues requiring food/feed safety assessment. None of the identified differences in the agronomic/phenotypic and compositional characteristics tested between maize DP910521 and its conventional counterpart needs further assessment except for the levels of iron in grain, which do not raise safety and nutritional concerns. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the Cry1B.34, PAT and PMI proteins as expressed in maize DP910521. The GMO panel finds no evidence that the genetic modification impacts the overall safety of maize DP910521. In the context of this application, the consumption of food and feed from maize DP910521 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that maize DP910521 is as safe as its conventional counterpart and non-GM maize varieties tested, and no post-market monitoring of food/feed is considered necessary. In the case of accidental release of maize DP910521 material into the environment, this would not raise environmental safety concerns. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of maize DP910521. The GMO Panel concludes that maize DP910521 is as safe as its conventional counterpart and the tested non-GM maize varieties with respect to potential effects on human and animal health and the environment.

KEYWORDS

Cry1B.34, DP910521, genetic engineering, GM, import and processing, maize (*Zea mays*), PAT, PMI

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SUMMARY

Following the submission of application GMFF-2021-2473 under Regulation (EC) No 1829/2003 from Corteva Agriscience LLC (referred to hereafter as 'the applicant'), the Panel on Genetically Modified Organisms of the European Food Safety Authority (referred to hereafter as 'GMO Panel') was asked to deliver a Scientific Opinion on the safety of genetically modified (GM) insect-resistant and herbicide-tolerant maize (*Zea mays* L.) DP910521 according to Regulation (EU) No 503/2013. The scope of application GMFF-2021-2473 is for import, processing and food and feed uses within the European Union (EU) of maize DP910521 and does not include cultivation in the EU.

In this scientific opinion, the GMO Panel reports on the outcome of its risk assessment of maize DP910521 according to the scope of the application GMFF-2021-2473. The GMO Panel conducted the assessment of maize DP910521 in line with the principles described in Regulation (EU) No 503/2013 and its applicable guidelines for the risk assessment of GM plants. The molecular characterisation data establish that maize DP910521 contains a single insert consisting of one copy of the *mo-pat*, *pmi* and *cry1B.34* expression cassettes. Updated bioinformatic analyses of the sequences encoding the newly expressed proteins and open reading frames (ORFs) present within the insert or spanning the junctions between the insert and genomic DNA do not raise any safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of the Cry1B.34, PAT and PMI proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-produced Cry1B.34, PAT and PMI proteins, indicate that these proteins are equivalent, and the microbial-derived proteins can be used in the safety studies.

Considering the selection of test materials, the field trial sites and the associated management practices and the agronomic–phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis. None of the identified differences in the agronomic, phenotypic and compositional characteristics tested between maize DP910521, and its conventional counterpart needs further assessment, except for iron in grains, which does not raise safety and nutritional concerns. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the Cry1B.34, PAT and PMI proteins as expressed in maize DP910521. The GMO Panel finds no evidence that the genetic modification impacts the overall safety of maize DP910521. In the context of this application, the consumption of food and feed from maize DP910521 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that maize DP910521 is as safe as its conventional counterpart and non-GM maize varieties tested, and no post-market monitoring of food/feed is considered necessary.

Considering the introduced traits, the outcome of the agronomic and phenotypic analysis and the routes and levels of exposure, maize DP910521 would not raise safety concerns in the case of accidental release of GM maize material into the environment. The post-market environmental monitoring (PMEM) plan and reporting intervals are in line with the intended uses of maize DP910521.

The GMO Panel considered the overall quality of the performed literature searches acceptable. Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issue pertaining to the intended uses of maize DP910521.

The GMO Panel concludes that maize DP910521 is as safe as its conventional counterpart and tested non-GM maize reference varieties with respect to potential effects on human and animal health and the environment.

1 | INTRODUCTION

The scope of the application GMFF-2021-2473 is for food and feed uses, import and processing of maize DP910521 and does not include cultivation in the European Union (EU). Maize DP910521 was developed to confer resistance against certain lepidopteran insect pests as well as tolerance to glufosinate herbicide.

1.1 | Background

On 27 June 2022, the European Food Safety Authority (EFSA) received from the Competent Authority of The Netherlands application GMFF-2021-2473 for authorisation of maize DP910521 (Unique Identifier DP-91Ø521-2), submitted by Corteva Agriscience LLC (hereafter referred to as 'the applicant') according to Regulation (EC) No 1829/2003.[1] Following receipt of application GMFF-2021-2473, EFSA informed EU Member States (MS) and the European Commission, and made the application available to them. Simultaneously, EFSA published a summary of the application.[2]

EFSA checked the application for compliance with the relevant requirements of Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013,[3] with the EFSA guidance documents, and, when needed, asked the applicant to supplement the initial application. On 21 December 2022, EFSA declared the application valid.

From validity date, EFSA and the Panel on Genetically Modified Organisms of the European Food Safety Authority (referred to hereafter as 'GMO Panel') endeavoured to respect a time limit of 6 months to issue a scientific opinion on application GMFF-2021-2473. Such time limit was extended whenever EFSA and/or GMO Panel requested supplementary information to the applicant. According to Regulation (EC) No 1829/2003, any supplementary information provided by the applicant during the risk assessment was made available to the EU Member States and European Commission (for further details, see the section 'Documentation', below). In accordance with Regulation (EC) No 1829/2003, EFSA consulted the nominated risk assessment bodies of EU Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC.[4] The EU Member States had 3 months from the date of validity to make their opinion on application GMFF-2021-2473 known as of that date of its validity.

1.2 | Terms of Reference as provided by the requestor

According to Articles 6 and 18 of Regulation (EC) No 1829/2003, EFSA and its GMO Panel were requested to carry out a scientific risk assessment of maize DP910521 in the context of its scope as defined in application GMFF-2021-2473.

In addition to the present scientific opinion, EFSA was also asked to report on the particulars listed under Articles 6(5) and 18(5) of Regulation (EC) No 1829/2003, but not to give an opinion on them because they pertain to risk management.

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant submitted a confidential and a non-confidential version of the dossier GMFF-2021-2473 following the EFSA requirements as detailed by EFSA (2021a, 2021b).

In accordance with Art. 38 of the Regulation (EC) No 178/2002[5] and taking into account the protection of confidential information and of personal data in accordance with Articles 39 to 39e of the same Regulation, the non-confidential version of the dossier was published in OpenEFSA.[6]

According to Art. 32c(2) of Regulation (EC) No 178/2002 and to the Decision of EFSA's Executive Director laying down the practical arrangements on pre-submission phase and public consultations,[7] EFSA carried out a public consultation on the non-confidential version of the dossier from 31 October to 21 November 2023 for which no comments were received.

The GMO Panel based its scientific assessment of maize DP910521 on the valid application GMFF-2021-2473, additional information provided by the applicant during the risk assessment, relevant scientific comments submitted by EU MS and relevant peer-reviewed scientific publications. As part of this comprehensive information package, the GMO Panel received

[1]Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1–23.

[2]https://open.efsa.europa.eu/dossier/GMFF-2021-2473?type=node&key=221589.

[3]Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. OJ L157, 8.6.2013, p. 1–48.

[4]Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 12.3.2001, p. 1–38.

[5]Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–48.

[6]https://open.efsa.europa.eu/questions/EFSA-Q-2022-00409.

[7]Decision https://www.efsa.europa.eu/sites/default/files/corporate_publications/files/210111-PAs-pre-submission-phase-and-public-consultations.pdf.

additional unpublished studies submitted by the applicant in order to comply with the specific provisions of Regulation (EU) No 503/2013. The list of these additional unpublished studies is provided in Appendix A.

2.2 | Methodologies

The GMO Panel conducted its assessment in line with the principles described in Regulation (EU) No 1829/2003, the applicable guidelines (i.e. EFSA GMO Panel, 2010a, 2011a, 2011b, 2015; EFSA Scientific Committee, 2011) and explanatory notes and statements (i.e. EFSA, 2010, 2014, 2017, 2018, 2019a, 2019b, 2021a, 2021b; EFSA GMO Panel, 2010b, 2018) for the risk assessment of GM plants.

For this application, in the context of the contracts OC/EFSA/GMO/2018/04, OC/EFSA/GMO/2020/01, OC/EFSA/GMO/2021/06, the contractors performed preparatory work for the evaluation of the applicant's literature search, the completeness and quality of DNA sequencing information, bioinformatic analyses on maize DP910521, respectively.

3 | ASSESSMENT

3.1 | Introduction

Maize DP910521 expresses the Cry1B.34 protein for control of certain lepidopteran insect pests, phosphinothricin acetyltransferase (PAT) protein that confers tolerance to the glufosinate ammonium-containing herbicides and the phosphomannose isomerase (PMI) protein that was used as a selectable marker during transformation.

The assessment of herbicide residues relevant for this application is in the remit of the EFSA Plant Health and Pesticides Residues Unit (EFSA, 2015).

3.2 | Systematic literature review[8]

The GMO Panel assessed the applicant's literature searches on maize DP910521, including a scoping review, according to the guidelines given in EFSA (2010, 2019b).

A systematic review as referred to in Regulation (EU) No 503/2013 has not been provided in support to the risk assessment of application GMFF-2021-2473. Based on the outcome of the scoping review, the GMO Panel agrees that there is limited value of undertaking a systematic review for maize DP910521at present.

The GMO Panel considered the overall quality of the performed literature searches acceptable. The literature searches identified four relevant peer-reviewed publications on maize DP910521 (Appendix B). Based on the relevant publications, the GMO Panel does not identify any safety issues pertaining to the intended uses of maize DP910521.

3.3 | Molecular characterisation[9]

3.3.1 | Transformation process and vector constructs

Maize DP910521 was developed by site-specific integration (SSI) using two sequential transformation steps:

1. Microprojectile co-bombardment and a CRISPR-Cas9-mediated targeted insertion process to allow the insertion of a 'landing pad' sequence, at a specific location of the maize genome (inbred PH184C line), using four plasmids (PHP71012, PHP70594, PHP21139 and PHP21875)
2. Microprojectile co-bombardment of a selected line containing the landing pad at the target location, to insert the intended expression cassettes into the landing pad in the maize genome, using four plasmids (PHP79620, PHP5096, PHP21875, PHP73572).

During the first transformation step, the expression of the zm84CR1 guide RNA leads the Cas9 protein to produce a double-stranded break in a targeted location in the maize genome. The break induces a homology-directed repair (HDR) mechanism, allowing a recombination between the zm-SEQ138 and zm-SEQ139 sequences from PHP71012 and the identical endogenous sequences present in the maize genome. As a result of the recombination event, the landing pad introduced in the plant genome a cassette containing a *loxP* site, the maize (*Z. mays*) ubiquitin (*ubiZM1*) 5'-UTR intron and promoter and the *nptII* gene with *pinII* terminator flanked by the flippase recombination target sites FRT1 and FRT87. Two more co-bombarded plasmids, PHP21139 and PHP21875, allowed the expression of the WUS2 protein and the ODP2

[8]Dossier: Part II – Section 7; additional information: 03/06/2023, 15/01/2024.
[9]Dossier: Part II – Section 1.2; additional information: 08/04/2023, 03/06/2023, 04/08/2023, 02/05/2024.

protein, respectively, to improve regeneration. The guide RNA, the *Cas9*, the *wus2* and the *odp2* genes were all transiently expressed, without integration in the plant genome. A maize line with the expected landing pad sequence and with no unintended plasmid DNA sequences was selected and used for the next step in the transformation process.

The plasmid PHP71012, used to insert the landing pad, contains one expression cassette, consisting of the following genetic elements:

- The *npt* II cassette consists of the promoter region from the maize ubiquitin gene 1 of *Zea mays* (*Z. mays* ubiZM1) including the 5′ untranslated region (5′ UTR) and intron, the coding sequence of the *npt* II gene from *Escherichia coli*, the terminator region from the *proteinase inhibitor II* (*pinII*) gene of *Solanum tuberosum (S. tuberosum)*. The *npt* II coding region and the terminator are flanked by the FRT1 and FRT87 recombination sites, intended to facilitate recombination after the second transformation step with plasmid PHP79620. The entire cassette, which contains a *lox*P site as well, is flanked by the zm-SEQ138 and zm-SEQ139 sequences, which are used to drive the insertion of the landing pad by homologous recombination in the homologous region in maize chromosome 1.

The plasmid PHP70594 contains two expression cassettes, consisting of the following genetic elements:

- The *cas9* cassette consists of the promoter region from the maize ubiquitin gene 1 of *Z. mays* (*ubiZM1*) including the 5′ untranslated region (5′ UTR) and intron; the exon 1 and exon 2 of the coding region of the *cas9* gene, interrupted by the LS1 intron of *S. tuberosum*. The coding region is flanked by the SV40 and VirD2 nuclear localisation signals (NLS) and the terminator of the *pin*II gene from *S. tuberosum*.
- The zm-84CR1 guide RNA cassette consists of the promoter region from the maize U6 polymerase III, the zm-84CR1 guide RNA from *Z. mays* and the terminator region of the U6 polymerase III from *Z. mays*.

The plasmid PHP21139 contains one expression cassette, consisting of the following genetic elements:

- The *zm-wus2* cassette consists of the promoter region of the *In2-2* gene of *Z. mays*, the coding sequence of the Wuschel2 (*wus2*) gene of *Z. mays* and the terminator region of the *In2-1* gene of *Z. mays*.

The plasmid PHP21875 contains one expression cassette, consisting of the following genetic elements:

- The *zm-odp2* gene cassette consists of the promoter region of the ubiquitin gene 1 of *Z. mays* (*ubiZM1*) including the 5′ untranslated region (5′ UTR) and intron, the coding sequence of the ovule development protein 2 (*odp2*) gene of *Z. mays* and the terminator region of the *pinII* gene of *S. tuberosum*.

In all the above-mentioned plasmids, the vector backbones contained elements necessary for the maintenance and selection of each plasmid in bacteria.

In the second step, a microprojectile co-bombardment was used to deliver plasmids PHP79620, PHP5096, PHP21875, PHP73572. The recombinant fragment region of plasmid PHP79620 comprises a total of three gene cassettes (*pmi, mo-pat* and *cry1B.34*), between the FRT1 and FRT87 sites, which are inserted into the landing pad of DP910521 maize genome by flippase-mediated recombination, replacing the *nptII* cassette introduced with the landing pad in the first step. The *mo-Flp* gene coding for the FLP recombinase is contained in plasmid PHP5096. Two more plasmids, PHP21875 and PHP73572, express the ODP2 protein and the WUS2 protein, respectively, to improve regeneration. The *mo-flp*, the *wus2* and the *odp2* genes are all transiently expressed, without integration in the plant genome.

The plasmid PHP79620, used to insert the desired cassettes in the landing pad, contains three expression cassettes, consisting of the following genetic elements:

- The *pmi* gene cassette consists of the *pmi* coding sequence of phosphomannose isomerase (*pmi*) gene from *Escherichia coli* including 5′ and 3′ UTRs, and the terminator region of the *pinII* gene of *S. tuberosum*. An additional terminator, the terminator region from *Z19* gene of *Z. mays* is also present.
- The *mo-pat* gene cassette consists of the promoter and intron region of the *os*-actin gene of *Oryza sativa*, the maize-optimised version of the pat coding sequence of the phosphinothricin acetyltransferase gene (*mo-pat*) from *Streptomyces viridochromogenes* and the CaMV 35S terminator. Two additional terminators are present: the terminator regions of the ubiquitin (*sb-ubi*) and γ-kafarin (*sb-gkaf*) genes of *Sorghum bicolor*.
- The *cry1B.34* gene cassette consists of two copies of the enhancer from the Mirabilis Mosaic Virus (MMV), the promoter region of the lamium distortion-associated virus (LLDAV), the intron region of the translation initiation factor 6 gene of *Z. mays*, the 5′ UTR of the extensin gene of *Z. mays*, the chimeric coding sequence of the *cry1B.34* gene, consisting of sequences of a cry1B-class gene, the *cry1Ca1* gene and the *cry9Db1* gene, the terminator region of the ubiquitin gene from *Oryza sativa*.

The PHP79620 plasmid also contains an *attB1*, *attB3* recombination sites and a *lox*P site.

The plasmid PHP5096 contains one expression cassette, consisting of the following genetic elements:

– The *mo-Flp* gene cassette consists of the promoter region of the ubiquitin gene 1 of *Z. mays* (*ubiZM1*) including the 5′ untranslated region (5′ UTR) and intron, maize-optimised coding region of the flippase (*Flp*) gene of *Saccharomyces cerevisiae*, and the terminator region of the *pinII* gene of *S. tuberosum*.

The plasmid PHP73572 contains one expression cassette, consisting of the following genetic elements:

– the *zm-wus2* gene cassette consists of the promoter region of the ubiquitin gene 1 of *Z. mays* (*ubiZM1*) including the 5′ untranslated region (5′ UTR) and intron, the coding sequence of the *Wuschel2 (wus2)* gene of *Z. mays* and the terminator region of the *pinII* gene of *S. tuberosum*.

The plasmid PHP21875 has also been used in the first transformation step, and it has been described above.

In all the above-mentioned plasmids, the vector backbones contained elements necessary for the maintenance and selection of the plasmid in bacteria.

3.3.2 | Transgene constructs in the GM plant

Molecular characterisation of maize DP910521 was performed by southern-by-sequencing (SbS) and junction sequence analysis (JSA) to determine insert copy number and to confirm the absence of plasmid backbone sequences and directed sequencing on PCR amplified fragments to determine size and organisation of the inserted sequences.

The EFSA GMO Panel assessed the sequencing data and found it compliant with the requirements listed in EFSA GMO Panel (2018), in terms of the approach, of the coverage and sensitivity.

NGS/JSA of the whole genome demonstrated that maize DP910521 contains a single insert, consisting of a single copy of the DNA regions deriving from PHP71012 and PHP79620. NGS/JSA also confirmed the absence of plasmid backbone sequences in the maize genome.

Sanger sequencing of PCR amplified fragments determined the nucleotide sequence of the entire maize DP910521 event consisting of 16,269 bp of the insert together with 1097 bp of the 5′ and 1054 bp of the 3′ flanking regions. The Sanger analysis revealed that the insert in maize DP910521 is identical to the intended landing pad sequence from plasmid PHP71012 and the DNA sequence from plasmid PHP79620.

The possible interruption of known endogenous maize genes by the insertion in maize DP910521 was evaluated by bioinformatic analyses of the pre-insertion locus and of the genomic sequences flanking the insert. The results of these analyses do not indicate the interruption of any known endogenous gene in maize DP910521.

The results of segregation (see Section 3.3.5) and bioinformatic analyses are compatible with a single insertion in the nuclear genome.

Updated bioinformatic analyses of the amino acid sequence of the newly expressed proteins reveal no significant similarities to toxins and allergens for Cry1B.34 and PAT, and an eight amino acids perfect match to a putative alpha-parvalbumin from *Rana* species for PMI which was previously assessed (EFSA GMO Panel, 2012). In addition, updated bioinformatic analyses of the newly created open reading frames (ORFs) within the insert and spanning the junctions between the insert and genomic DNA, indicated that six ORFs (DP910521_224, DP910521_361, DP910521_364, DP910521_370, DP910521_729 and DP910521_889) exceeded the allergenicity assessment threshold of 35% identity using an 80 amino acid sliding window approach and two ORFs (DP910521_209 and DP910521_728) contains an eight amino acid perfect match to allergens. However, three of these ORFs (DP910521_728, DP910521_729 and DP910521_889) are predicted on the complementary strand and lack promoter and start codons, while the remaining five (DP910521_209, DP910521_224, DP910521_361, DP910521_364, DP910521_370) are predicted in coding region of the NEPs but in a different reading frame. In conclusion, these analyses indicated that the expression of any ORF showing significant similarities to toxins or allergens in maize DP910521 is unlikely.

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination (HR), the applicant performed a sequence identity analysis for maize DP910521 to microbial DNA. The likelihood and potential consequences of plant-to-bacteria gene transfer are described in Section 3.6.1.2.

3.3.3 | Protein characterisation and equivalence

Maize DP910521 expresses three new proteins: Cry1B.34 protein for protection against certain lepidopteran insect pests, phosphinothricin acetyltransferase (PAT) protein for tolerance to glufosinate herbicide and phosphomannose isomerase (PMI) protein that was used as a selectable marker during transformation. Given the technical restraints in producing large enough quantities from plants for protein characterisation, these proteins were recombinantly produced in *Escherichia coli*. A set of biochemical methods was employed to demonstrate the equivalence between the maize and *E. coli*-derived Cry1B.34, PAT and PMI. Purified proteins from these two sources were characterised and compared in terms of their biochemical, structural and functional properties.

Cry1B.34 protein characterisation and equivalence

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis showed that both plant and microbe-produced Cry1B.34 proteins had the expected molecular weight of ~129 kDa and were comparably immunoreactive to Cry1B.34 protein-specific antibodies. Glycosylation analysis demonstrated that none of the Cry1B.34 proteins were glycosylated. Amino acid sequence analysis of the plant-derived Cry1B.34 protein by mass spectrometry (MS) methods showed that the protein matched the deduced sequence as defined by the *cry1b.34* gene. These sequence analysis data were consistent with the previously analysed microbe-produced Cry1B.34. In addition, the MS data showed that the N-terminal methionine of the plant-produced Cry1B.34 protein was truncated. Such modifications are common in eukaryotic proteins (e.g. Polevoda & Sherman, 2000). Functional equivalence was demonstrated by an insect bioassay which showed that plant and microbe-derived Cry1B.34 proteins had comparable insecticidal activity.

The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-produced Cry1B.34 proteins indicate that these two proteins are equivalent, and the microbial-derived protein can be used in the safety studies.

PAT protein characterisation

SDS-PAGE and western blot analysis showed that both plant and microbe-produced PAT proteins had the expected molecular weight of ~21 kDa and were comparably immunoreactive to PAT protein-specific antibodies. Glycosylation analysis demonstrated that none of the PAT proteins were glycosylated. Amino acid sequence analysis of the plant-derived PAT protein by MS methods showed that the protein matched the deduced sequence as defined by the *pat* gene. These sequence analysis data were consistent with the previously analysed microbe-produced PAT. In addition, the MS data showed that the N-terminal methionine of the plant-produced PAT protein was truncated. Such modifications are common in eukaryotic proteins (e.g. Polevoda & Sherman, 2000).

PMI protein characterisation

SDS-PAGE and western blot analysis showed that both plant and microbe-produced PMI proteins had the expected molecular weight of ~43 kDa and were comparably immunoreactive to PMI protein-specific antibodies. Glycosylation detection analysis demonstrated that none of the PMI proteins were glycosylated. Amino acid sequence analysis of the plant-derived PMI protein by MS methods showed that the protein matched the deduced sequence as defined by the *pmi* gene. These sequence analysis data were consistent with the previously analysed microbe-produced PMI. In addition, the MS data showed that the N-terminal methionine of the plant-produced PMI protein was acetylated. Such modifications are common in eukaryotic proteins (e.g. Polevoda & Sherman, 2000).

3.3.4 | Information on the expression of the insert

Protein levels of Cry1B.34, PAT and PMI were analysed by an enzyme-linked immunosorbent assay (ELISA) in material harvested in a field trial across six locations in the United States and Canada during the 2020 growing season. Analysed samples included leaf (BBCH 16, BBCH 19, BBCH 63–65 and BBCH 85), root (BBCH 19, BBCH 63–65 and BBCH 85), pollen (BBCH 63–65), stalk (BBCH 63–65), forage (BBCH 85) and grains (BBCH 87–99) from plants treated and not treated with glufosinate. The mean values and standard deviations of protein expression levels in grain ($n = 24$), forage ($n = 24$) and pollen ($n = 24$) of the Cry1B.34, PAT and PMI proteins used to estimate human and animal dietary exposure (see Section 3.5.4.2) are reported in Table 1.

TABLE 1 Mean values ($n = 24$) and standard deviation of newly expressed protein in grains [ng/mg dry weight (dw) and ng/mg fresh weight (fw)] pollen and forage (ng/mg dw) from maize DP910521.

	Glufosinate treatment			
	Not treated		Treated	
Tissue	ng/mg dry weight (dw)	ng/mg fresh weight (fw)	ng/mg dry weight (dw)	ng/mg fresh weight (fw)
Grains (BBCH 87–99)				
Cry1B.34	29[a] ± 15[b] (8.4-58)[c]	24 ± 13 (7.1–49)	32 ± 16 (9.5–68)	27 ± 13 (8.0–57)
PAT	14 ± 4.8 (7.4–25)	12 ± 4.0 (6.2–21)	16 ± 4.6 (8.3–26)	13 ± 3.9 (7.0–22)
PMI	10 ± 3.5 (4.4–17)	8.4 ± 2.9 (3.7–14)	10 ± 2.7 (6.3–17)	8.4 ± 2.3 (5.3–14)
Forage (BBCH 85)				
Cry1B.34	450 ± 130[d] (200–710)		390 ± 91 (250–540)	
PAT	120 ± 45 (50–230)		120 ± 52 (63–270)	
PMI	25 ± 8.2 (16–51)		27 ± 11 (20–67)	

(Continues)

TABLE 1 (Continued)

	Glufosinate treatment			
	Not treated		Treated	
Tissue	ng/mg dry weight (dw)	ng/mg fresh weight (fw)	ng/mg dry weight (dw)	ng/mg fresh weight (fw)
Pollen (BBCH 63–65)				
Cry1B.34	< LOQ[e] (< LOQ-1.7)		< LOQ[e] (< LOQ-0.58)	
PAT	67 ± 11 (54–99)		68 ± 10 (51–99)	
PMI	23 ± 5.3 (18–39)		24 ± 4.6 (17–36)	

[a]Mean value.
[b]Standard deviation.
[c]Range.
[d]*N* = 23 for Cry1B.34 in forage not treated.
[e]21 out of 24 samples in pollen not treated and 17 out of 24 samples in pollen treated were below the LLOQ (LOQ = 0.28 ng/mg). A value equal to half the LLOQ was assigned to these samples to calculate the mean.

3.3.5 | Inheritance and stability of inserted DNA

Genetic stability of maize DP910521 insert was assessed by Southern blot analysis of genomic DNA from five generations (T1, BC1, BC1F1, BC1F2, BC1F3) while inheritance pattern was assessed by quantitative polymerase chain reaction (qPCR)-based segregation analysis and phenotypic analysis (resistance to glufosinate) from five generations: four segregating generations (F1, F2, BC1 and BC1S1) and one non-segregating generation (BC1S3).

The results support the presence of a single insertion, segregating in a Mendelian fashion.

3.3.6 | Conclusion on molecular characterisation

The molecular characterisation data establish that maize DP910521 contains a single insert consisting of one copy of the *mo-pat, pmi and cry1B.34* expression cassettes. Bioinformatic analyses of the sequences encoding the newly expressed proteins and other ORFs within the insert or spanning the junctions between the insert and genomic DNA do not raise any safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of the Cry1B.34, PAT and PMI proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-produced Cry1B.34 proteins indicate that these proteins are equivalent, and the microbial-derived protein can be used in the safety studies.

3.4 | Comparative analysis[10]

3.4.1 | Overview of studies conducted for the comparative analysis

Application GMFF-2021-2473 presents data on agronomic and phenotypic characteristics, as well as on forage and grain composition of maize DP910521 (Table 2). In addition, the application contains a germination and viability study of maize line containing event DP910521 (see Appendix A).

TABLE 2 Main comparative analysis studies to characterise maize DP910521 provided in the application GMFF-2021-2473.

Study focus	Study details	Comparator	Non-GM reference varieties
Agronomic and phenotypic analysis	Field study, U.S and Canada, 2020, eleven sites[a]	PH47K2 × PH184C	20[b]
Compositional analysis	Field study, U.S and Canada, 2020, eight sites[c]		

Abbreviation: GM, genetically modified.
[a]Two field trials were located in each of Iowa and Illinois (United States); one field trial was located for each of the following States: Indiana, Minnesota, Nebraska, Pennsylvania, Texas and Wisconsin (US) and Ontario (Canada). An additional site present in Ontario (Canada) was excluded from the statistical analysis due to soil compaction resulting from field activities during the previous season.
[b]Non-GM hybrid maize with their corresponding comparative relative maturity indicated in brackets were P0506 (105); XL5513 (105); P0574 (106); PB5646 (106); P0760 (107); G07F23 (107); 207–27 (107); BK5883 (108); P0843 (108); BKXL-5858 (108); 209–50 (109); P0928 (109); P0993 (109); XL5939 (109); 6046 (110); XL5828 (110); P1093 (110); DKC60-84 (110); G10T63 (110) and BK6076 (110).
[c]Two field trials were located in Illinois (US); one field trial was located in each of the following States: Iowa, Nebraska, Pennsylvania, Texas, and Wisconsin (US) and Ontario (Canada).

[10]Dossier: Part II – Section 1.3; additional information: 22/02/2023, 03/06/2023.

3.4.2 | Experimental field trial design and statistical analysis

At each field trial site, the following materials were grown in a randomised complete block design with four replicates: maize DP910521 not exposed to the intended herbicide, maize DP910521 exposed to the intended herbicide, the comparator PH47K2 × PH184C and four non-GM reference varieties.

The agronomic, phenotypic and compositional data were analysed as specified by EFSA GMO Panel (2010b, 2011a). This includes, for each of the two treatments of maize DP910521, the application of a difference test (between the GM maize and the non-GM comparator) and an equivalence test (between the GM maize and the set of non-GM reference varieties). The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).[11]

3.4.3 | Suitability of selected test materials

3.4.3.1 | *Selection of the test materials*

As described in Section 3.3.1, inbred line PH184C was transformed to obtain line DP910521, which was then crossed with the non-GM inbred line PH47K2 to produce the hybrid maize DP910521[12] used to conduct the agronomic and phenotypic and the compositional assessment.

The comparator used in the field trials is the non-GM maize hybrid PH47K2 × PH184C, which is isogenic to hybrid maize DP910521 (as documented by the pedigree) and is considered to be the conventional counterpart.

Hybrid maize DP910521 and the conventional counterpart (PH47K2 × PH184C), both with a comparative relative maturity (CRM) of 108, are considered appropriate for growing in a range of environments across North America, where the comparative field trials were conducted.

The non-GM reference varieties (see Table 2) with a CRM ranging from 105 to 110 were selected by the applicant and, at each selected site, four of them were tested. On the basis of the information provided on relative maturity classes and year of commercialisation, the GMO Panel considers the selected non-GM reference varieties appropriate for the comparative assessment.

3.4.3.2 | *Seed production and quality*

Seeds of maize DP910521 and the conventional counterpart used in the 2020 field trials (see Table 2) were produced, harvested and stored under similar conditions, before being sown in the field trial sites. The seed lots were verified for their identity via event-specific quantitative PCR analysis.

The germination capacity of GM maize DP910521 and the conventional counterpart was tested under warm and cold temperature conditions.[13] Germination capacity of the GM maize DP910521 was compared with the one of its conventional counterpart. The results of these studies indicate that the seed germination of maize DP910521 was not different than that of its conventional counterpart.[14] The GMO Panel considers that the starting seed used as test material in the agronomic, phenotypic and compositional studies was of suitable quality.

3.4.3.3 | *Conclusion on suitability*

The GMO Panel is of the opinion that the maize DP910521, the conventional counterpart and the non-GM reference hybrids were properly selected and are of adequate quality. Therefore, the test materials are considered suitable for the comparative analysis.

[11] In detail, the four outcomes are category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence) and category IV (indicating non-equivalence).

[12] For the agronomic, phenotypic and compositional analysis, hybrid maize DP910521 refers to the event obtained crossing inbred line DP910521 in PH184C with the inbred line PH47K2.

[13] Warm temperature condition corresponds to ~25°C and 90% relative humidity for 7 days and cold to 10°C and 90% relative humidity for 7 days followed by 5 days at 25°C and 90% relative humidity.

[14] GM hybrid showed a mean germination of 98% and 97% while the conventional counterpart showed a mean of 97% and 98% under warm and cold temperature conditions, respectively.

3.4.4 | Representativeness of the receiving environments

3.4.4.1 | *Selection of field trial sites*

The selected field trials sites were located in commercial maize-growing regions of United States of America and Canada. The soil and climatic characteristics of the selected fields[15] correspond to optimal, near-optimal and suboptimal conditions for maize cultivation (Sys et al., 1993).

The GMO Panel considers that the selected sites, including the subset chosen for the compositional analysis, reflect commercial maize-growing regions in which the test materials are likely to be grown.

3.4.4.2 | *Meteorological conditions*

Maximum and minimum mean temperatures and sums of precipitation were provided for each site on a weekly basis.

Some exceptional weather conditions were reported at six of the selected sites.[16] However, due to the lack of major impacts on plant growth at these sites, the GMO Panel considers that the exceptional weather conditions did not invalidate the use of data from the field trial sites for the comparative analysis.

3.4.4.3 | *Management practices*

The field trials included plots containing maize DP910521, plots with the conventional counterpart and plots with non-GM maize reference varieties, managed according to local agricultural practices.

In addition, the field trials included plots containing maize DP910521 managed following the same agricultural practices, but conventional herbicides were replaced with the intended glufosinate ammonium-containing herbicide that was applied at the BBCH 14 growth stage.

The GMO Panel considers that the management practices, including sowing, harvesting and application of plant protection products, were appropriate for the selected receiving environments.

3.4.4.4 | *Conclusion on representativeness*

The GMO Panel concludes that the geographical locations, soil and climatic characteristics, meteorological conditions and management practices of the field trial sites are typical for receiving environments where the tested materials could be grown.

3.4.5 | Agronomic and phenotypic analysis

Eleven agronomic and phenotypic endpoints[17] plus information on abiotic stressors, disease incidence and arthropod damage were collected from the field trial sites (see Table 2). The endpoints ear count and dropped ears were not analysed with formal statistical methods because of lack of variability in the data.

The statistical analysis (Section 3.4.2) was applied to the remaining nine endpoints, with the following results:

- For maize DP910521 (not treated with the intended herbicide), the test of difference identified statistically significant differences with the conventional counterpart for early stand count, days to flowering, plant height, final stand count and 100-kernel weight. All these endpoints fell under equivalence category I or II.
- For maize DP910521 (treated with the intended herbicide), the test of difference identified statistically significant differences with the conventional counterpart for early stand count, days to flowering, plant height, days to maturity and 100-kernel weight. All these endpoints fell under equivalence category I or II.

[15]Soil types of the field trials were sandy clay loam, silty clay loam, silty loam, loamy sand, loam and clay loam; soil organic matter ranged from 0.9% to 2.4%; pH ranged from 6.0 to 7.7; average temperatures and sum of precipitations during the usual crop growing season ranged, respectively, from 16.3°C to 23.0°C and from 56 mm to 763 mm.

[16]Strong wind was registered at one field trial in Iowa; windstorms in Iowa, Illinois and Minnesota; excessive rainfall in Indiana; saturated soils in Minnesota and extreme heat and high rainfall in Ontario (Canada).

[17]Early stand count, days to flowering, plant height, days to maturity, lodging, final stand count, ear count, dropped ears, yield, harvest grain moisture and 100-kernel weight.

3.4.6 | Compositional analysis

Maize DP910521 forage and grain harvested from eight sites (Table 2) were analysed for 80 constituents (10 in forage and 70 in grain), including those recommended by OECD (OECD, 2002). The statistical analysis was not applied to eight grain constituents because their concentration in more than half of the samples was below the limit of quantification.[18]

The statistical analysis was applied to a total of 72 constituents (10 in forage[19] and 62 in grain[20]); a summary of the outcome of the test of difference and the test of equivalence is presented in Table 3:

- For maize DP910521 not treated with the intended herbicide, statistically significant differences with the conventional counterpart were found for 11 endpoints (four in forage and seven in grain). All these endpoints for which significant differences were found between the GM maize and the conventional counterpart fell under equivalence category I or II.
- For maize DP910521 treated with the intended herbicide, statistically significant differences with the conventional counterpart were found for 15 endpoints (three in forage and 12 in grain). All these endpoints for which significant differences were found between the GM maize and the conventional counterpart fell under equivalence category I or II, except for iron in grain which fell under equivalence category III.

TABLE 3 Outcome of the comparative compositional analysis in forage and grain for maize DP910521. The table shows the number of endpoints in each category.

		Test of difference[a]			
		Not treated[b]		Treated[b]	
		Not different	Significantly different	Not different	Significantly different
Test of equivalence[c]	Category I/II	56	11[d]	51	14[d]
	Category III/IV	3[e]	–	4[e]	1[f]
	Not categorised	2[g]	–	2[g]	–
	Total endpoints	72		72	

[a]Comparison between maize DP910521 and its conventional counterpart.

[b]Treated/not treated with the intended herbicide.

[c]Four different outcomes: category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence). Not categorised means that the test of equivalence was not applied because of the lack of variation among the non-GM reference varieties.

[d]Endpoints with significant differences between maize DP910521 and its conventional counterpart and falling under equivalence category I–II. For forage, not treated only: crude protein, ash, carbohydrates, phosphorous; treated only: crude fibre, ADF, NDF. For grains, not treated only: heptadecanoic acid (C17:0), stearic acid (C18:0), lignoceric acid (C24:0), magnesium. Treated only: crude fat, palmitoleic acid (C16:1), tyrosine, copper, zinc, thiamine, niacin, p-coumaric acid. Both treated and not treated: moisture, ash, phytic acid.

[e]Endpoints in grain with no significant differences between maize DP910521 and its conventional counterpart and falling under equivalence category III/IV: treated only: tryptophan; both for treated and not treated: arachidic acid (C20:0), eicosenoic acid (C20:1), behenic acid (C22:0).

[f]Endpoint with significant differences between the maize DP910521 and its conventional counterpart and falling in equivalence category III: treated only: iron in grain. Estimated means are reported for these endpoints in Table 4.

[g]Endpoints not categorised for equivalence and without significant differences between the maize DP910521 and its conventional counterpart: in grain, both treated and not treated: sodium, folic acid.

The GMO Panel assessed all significant differences between maize DP910521 and its conventional counterpart, taking into account the potential impact on plant metabolism and the natural variability observed for the set of non-GM reference varieties. Quantitative results for the endpoints showing significant differences between maize DP910521 and its conventional counterpart and falling under category III/IV are presented in Table 4.

[18]Palmitoleic acid (C16:1), heptadecanoic acid (C17:0), behenic acid (C22:0), copper, sodium, β-carotene, thiamine, raffinose.

[19]Moisture, crude protein, crude fat, ash, carbohydrates, crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), calcium and phosphorus.

[20]Proximate and fibre content (ash, carbohydrates, crude fat, crude fibre, crude protein, moisture, acid detergent fibre (ADF), neutral detergent fibre (NDF) and total dietary fibre (TDF)), minerals (calcium, iron, magnesium, manganese, phosphorus, potassium, zinc), vitamins (α-tocopherol, γ-tocopherol, total tocopherols, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid), amino acids (alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine), fatty acids (lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), heptadecenoic acid (C17:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3), arachidic acid (C20:0), eicosenoic acid (C20:1), eicosadienoic acid (C20:2), lignoceric acid (C24:0)) and other compounds (p-coumaric acid, ferulic acid, furfural, inositol, phytic acid, trypsin inhibitor).

TABLE 4 Quantitative results (estimated means and equivalence limits) for compositional endpoints in maize DP910521 grain that are further assessed based on the results of the statistical analysis.

	Maize DP910521[a]			Non-GM reference varieties	
Endpoint	**Not treated**	**Treated**	**Conventional counterpart**	**Mean**	**Equivalence limits**
Iron (mg/Kg dw)	17.8	18.7*	17.8	16.4	14.5–18.3

Abbreviations: dw, dry weight; Treated, treated with the intended herbicide; not treated: treated only with conventional herbicides (see Section 3.4.4.3).

[a]For the maize DP910521, significantly different values are marked with an asterisk, while the outcomes of the test of equivalence are differentiated by greyscale backgrounds: white (equivalence category I) and light grey (equivalence category III).

3.4.7 | Conclusion on comparative analysis

Considering the selection of test materials, the field trial sites and the associated management practices and the agronomic–phenotypic characterisation as an indicator of the overall field trial quality, the GMO Panel concludes that the field trials are appropriate to support the comparative analysis.

Taking into account the natural variability observed for the set of non-GM reference varieties, the GMO Panel concludes that:

- None of the differences identified in agronomic and phenotypic characteristics between maize DP910521 and the conventional counterpart needs further assessment regarding their potential environmental impact.
- None of the differences identified in forage and grain composition between the maize DP910521 and the conventional counterpart needs further assessment regarding food and feed safety except for the levels of iron in grain, which is further assessed in Section 3.5.

3.5 | Food/feed safety assessment[21]

3.5.1 | Overview of overarching information for food/feed assessment

3.5.1.1 | *Compositional analysis*

The compositional analysis of maize DP910521 and the conventional counterpart provided by the applicant and assessed by the GMO Panel is described in Section 3.4.6.

3.5.1.2 | *Newly expressed proteins*

Three proteins, Cry1B.34, PAT and PMI, are newly expressed in maize DP910521. The Cry1B.34 protein has not been previously assessed by the GMO Panel and it will be the focus of the assessment in this section. The PAT and PMI proteins have been previously assessed by the GMO Panel and no safety concerns for humans and animals (i.e. farmed and companion animals) were identified (EFSA GMO Panel, 2012, 2017b, 2024).

3.5.1.2.1 | *Molecular characterisation*

The protein characterisation of the newly expressed Cry1B.34 protein provided by the applicant and assessed by the GMO Panel is described in Section 3.3.3.

3.5.1.2.2 | *History of safe use for consumption as food/feed of the NEPs*

a. Information on the source organism

The Cry1B.34 protein's gene source organism is a ubiquitous soil bacterium (*B. thuringiensis*) and has been reported to protect plants by producing Bt toxins that inhibit the growth of insects and nematodes. Furthermore, Bt microbials are used as sprayed pesticides for pest control in agriculture.

b. Information on structure, function and mode of action

The insecticidal protein Cry1B.34 confers protection against certain lepidopteran insect pests when expressed in plants by causing disruption of the midgut epithelium. It is reported that the gastrointestinal tract of mammals, including humans, lacks receptors with high-specific affinity to Cry proteins (Koch et al., 2015; Jurat-Fuentes & Crickmore, 2017).

[21]Dossier: Part I – Section 1.4, 1.5, 1.6; additional information: 22/02/2023, 08/04/2023, 30/06/2023, 04/08/2023, 18/10/2023, 15/01/2024, 24/01/2024, 27/02/2024, 02/04/2024, 02/05/2024.

c. Information on identity/homology of the NEP to other proteins in conventional food and feed sources

The GMO Panel is not aware of any information on identity/homology of Cry1B.34 protein to other proteins in conventional food and feed sources.

d. Overall conclusion of the history of safe use

The GMO Panel considers the information above as not sufficient to duly document the history of safe use for consumption of the newly expressed Cry1B.34 protein.

3.5.1.2.3 | *Stability of the NEPs*

Protein stability is one of several relevant parameters to consider in the weight-of-evidence approach in protein safety assessment (EFSA GMO Panel, 2010c, 2011a, 2017a, 2021). The term protein stability encompasses several properties such as thermal stability, pH-dependent stability, proteolytic stability and physical stability (e.g. tendency to aggregate), among others (Li et al., 2019). It has been shown, for example, that when characteristics of known food allergens are examined, one of the most prominent traits attributed to food allergens is protein stability (Breiteneder & Mills, 2005; Costa et al., 2022; Foo & Mueller, 2021; Helm, 2001).

a. Effect of temperature and pH on NEPs

The effects of temperature and pH on PAT and PMI proteins as expressed in maize DP910521 were previously assessed by the GMO Panel (EFSA GMO Panel, 2012, 2017b, 2024). The applicant provided studies on the Cry1B.34 protein as expressed in maize DP910521. Cry1B.34 protein samples were incubated for ~ 30 min at 25°C, 50°C, 75°C and 95°C followed by a functional activity bioassay. These studies showed that the Cry1B.34 protein was inactive after incubation at temperatures ≥ 75°C.

In relation to the effect of pH on the Cry1B.34 protein, the molecular mass (~ 129 kDa) of the protein was unchanged at pH 1.2.

b. In vitro protein degradation by proteolytic enzymes

In vitro protein degradation studies on PAT and PMI proteins as expressed in maize DP910521 were previously evaluated by the GMO Panel (EFSA GMO Panel, 2012, 2017b, 2024).

Furthermore, the applicant provided information on in vitro protein degradation (i.e. resistance to pepsin in solutions at pH ~ 1.2) of the Cry1B.34 protein from a microbial recombinant system. The integrity of the test protein in samples of the incubation mixture taken at various time points was analysed by SDS-PAGE followed by protein staining or by Western blotting. The Cry1B.34 protein was degraded by pepsin within 0.5 min of incubation. A peptide fragment of ~ 20 kDa was also observed but degraded within 5 min. In addition, transient peptide fragments of low molecular weight were observed at different time points by SDS-PAGE. Furthermore, the applicant provided a study where the Cry1B.34 protein was subjected to a sequential digestion, pepsin followed by pancreatin. The transient peptide fragments seen in the pepsin analysis were degraded within 0.5 min of exposure to pancreatin when analysed by SDS-PAGE. The sequential addition of digestive enzymes – gastric digestion conditions followed by an intestinal in vitro digestion – has been proposed as part of several alternative protocols to the classical pepsin resistance test to simulate more closely, within the inherent limitations of in vitro models, the physiological conditions of gastrointestinal digestion (EFSA GMO Panel, 2021). This is in line with Codex Alimentarius which indicates that alternative in vitro digestion protocols may be used, where adequate justification is provided (Codex Alimentarius, 2009).

3.5.1.2.4 | *Synergistic or antagonistic interactions*

The potential for a functional interaction among the Cry1B.34, PAT and PMI proteins has been assessed with regard to human and animal health. Based on current scientific knowledge on the biological function of the three proteins (Table 5), no synergistic or antagonistic interactions between these three proteins which could raise safety concerns for food and feed from maize DP910521 are expected.

TABLE 5 Intended effects of the three NEPs in maize DP910521.

Protein	Intended effect in GM plant
Cry1B.34	The Cry1B.34 protein confers resistance to certain lepidopteran pests
PAT	The PAT protein confers tolerance to glufosinate-ammonium-based herbicides acting by acetylation of glufosinate-ammonium
PMI	The PMI (phosphomannose isomerase) protein is used as a selectable marker and plays a role in the metabolism of mannose, which normally inhibits root growth, respiration and germination in plants. Transformed cells expressing PMI are able to utilise mannose as a carbon source

3.5.1.3 | *Effect of processing*

Maize DP910521 will undergo existing production processes used for conventional maize. No novel production process is envisaged. Based on the outcome of the comparative assessment, processing of the GM maize into food and feed products is not expected to result in products being different from those of conventional non-GM maize varieties.

3.5.2 | Toxicology

The strategies to assess the toxicological impact of any changes on the whole food and feed resulting from the genetic modification focus on the assessment of (i) newly expressed proteins; (ii) new constituents other than NEPs; (iii) altered levels of food and feed constituents; and (iv) the whole genetically modified food and feed.

3.5.2.1 | *Assessment of newly expressed proteins*

The PAT and PMI proteins have been previously assessed by the GMO Panel in the context of other applications (EFSA GMO Panel, 2012, 2017b, 2024) and no safety concerns for humans and animals (i.e. farmed and companion animals) were identified. Updated bioinformatic analyses revealed no similarities of these proteins with known toxins. The GMO Panel is not aware of any new information that would change the previous conclusion on the safety of PAT and PMI proteins.

A weight-of-evidence approach was followed by the GMO Panel to assess the toxicological profile of the newly expressed Cry1B.34 protein, taking into account all of the information relevant for the hazard assessment, including molecular characterisation, substrate specificity, history of safe use for consumption as food and feed of the NEPs, stability of the NEPs and synergistic or antagonistic interactions (Section 3.5.1.2), updated bioinformatic analyses for similarity to toxins and in vivo toxicity studies.

3.5.2.1.1 | *Bioinformatic analyses*

Updated bioinformatic analyses of the amino acid sequences of Cry1B.34 protein revealed no relevant similarities to known toxins.

3.5.2.1.2 | *In vivo toxicity studies*

For the assessment of the Cry1B.34 protein, the applicant provided a 28-day toxicity study and an acute toxicity study. The outcome of the in vivo toxicity studies is described below.

Acute toxicity study with Cry1B.34 protein

An acute toxicity study in CD1 mice administered the *E. Coli*-produced Cry1B.34 protein by gavage at the dose of 5000 mg/kg (bw) showed no adverse effects.

28-day repeated dose toxicity study with Cry1B.34 protein

The 28-day repeated dose toxicity study in mice with Cry1B.34 protein was conducted in accordance with OECD TG 407 (2008) and to the principles of good laboratory practice.

Groups of Crl:CD-1 mice (10/sex per group), ~8-week-old at the start of dosing were allocated to three groups. Groups were administered diets containing, respectively, the test substance (Cry1B.34 protein) at targeted nominal doses of 1000 or 300 mg/kg body weight (bw) per day (high and low Cry1B.34 protein groups); or a basal diet (control group). Additional 10 mice/sex/group were used to investigate coagulation parameters (satellite animals).

The test substance used in this study was produced by a recombinant system and contained about 0.77 mg Cry1B.34 mg lyophilised powder. The amino acid sequence analysis of the *E. coli*-produced Cry1B.34 used in this 28-day toxicity study matched the deduced sequence as defined by the *cry1B.34* gene. This protein had the expected molecular weight, was not glycosylated, and showed functional activity.

In-life procedures, observations and terminal procedures were conducted in accordance with TG 407 (OECD, 2008), except for satellite animals that were not subjected to some in life procedure (ophthalmology, functional observational battery, motor activity), organ weights, and macroscopic and microscopic examinations.

The GMO Panel noted that animals were singly housed, and considered the justification provided by the applicant acceptable. Deviations to the protocol reported in the study were considered minor deviations with no impact on the study results.

Based on the results of concentration analysis by ELISA, the applicant confirmed the expected dietary concentrations (1.95, 6.5 g/kg diet). The results of the test diet analyses indicated that they were homogeneous and exhibited acceptable stability.

An appropriate range of statistical tests was performed on the results of the study and a detailed description of the methodology and of statistically significant findings identified in mice is reported in Appendix C.

There were no test diet-related incidents of mortality or clinical signs. No test diet-related adverse findings were identified in any of the investigated parameters. A small number of statistically significant findings were noted, but these were not considered adverse effects of treatment for one or more of the following reasons:

- were within the normal variation[22] for the parameter in mice of this age;
- were of small magnitude;
- were identified at only a small number of time intervals with no impact on the overall value;
- exhibited no consistent pattern with related parameters or end-points;
- exhibited no consistency with increasing dose levels.

No gross pathology findings related to the administration of the test diet were observed at necropsy, and the microscopic examinations of a wide range of organs and tissues did not identify relevant differences in the incidence or severity of the histopathology findings related to the administration of the test diet compared to the control group.

The GMO Panel concludes that no adverse effects were observed in mice in this 28-day toxicity study on *E. coli*-produced Cry1B.34 protein, at nominal dietary exposures up to 1000 mg/kg bw per day.

Conclusion of the toxicological assessment of the Cry1B.34 protein

Based on the above information, the GMO Panel did not identify indications that the Cry1B.34 protein raises food and feed safety concerns in humans and animals.

3.5.2.2 | *Assessment of new constituents other than NEPs*

Based on the outcome of the studies considered in the comparative analysis and molecular characterisation, no new constituents other than the newly expressed proteins have been identified in grain and forage from maize DP910521. Therefore, no further food/feed safety assessment of components other than the newly expressed proteins is required.

3.5.2.3 | *Assessment of altered levels of food and feed constituents*

Based on the outcome of the studies considered in the comparative analysis and molecular characterisation, no altered levels of food and feed constituents have been identified in grain and forage of maize DP910521, except for iron in grain. This change is considered not to represent a toxicological concern, considering the biological role of the affected constituent and the magnitude of the change. Therefore, no further toxicological assessment is needed. Further information on the relevance of this finding is provided in Section 3.5.5.

3.5.2.4 | *Testing of the whole genetically modified food and feed*

Based on the outcome of the molecular characterisation and comparative analysis assessment, no compositional modifications or indication of possible unintended effects relevant to food and feed safety have been identified for maize DP910521. Therefore, animal feeding studies with food/feed derived from maize DP910521 are not considered necessary by the GMO Panel (EFSA GMO Panel, 2011a). In accordance with Regulation (EU) No 503/2013, the applicant provided a 90-day feeding study in rats fed with diets containing grains derived from maize DP910521.

In this study, pair-housed Crl:CD(SD) rats (16 per sex per group, 2 rats per cage) were allocated to six groups, using a randomised complete block design with eight replications per sex.

Groups were fed diets containing maize DP910521 grains from plants treated with the intended herbicide (glufosinate-ammonium containing herbicides) at 50% and 33% of inclusion level (the latter supplemented with 17% of the conventional counterpart), the conventional counterpart (inclusion level 50%) and the reference varieties (BK5883, P0843, and P0993) (inclusion level 50%).

The study was adapted from OECD test guideline 408 (OECD, 2018), aligned with EFSA Scientific Committee guidance (EFSA Scientific Committee, 2011) and complied with the principles of good laboratory practice (GLP).

The stability of the test and control materials was not analytically verified; however, it was confirmed that the diet was used within the expiry date declared by the diet manufacturer. The GMO Panel considered this justification acceptable evidence that the constituents of the diets would be stable for the duration of the treatment. Furthermore, diet preparation procedures and regular evaluations of the mixing methods guaranteed the homogeneity and the proper concentration of the test or control substances in them.

[22]Although animals used in a toxicology study are of the same strain, from the same supplier and are closely matched for age and body weight at the start of the study, they exhibit a degree of variability in the parameters investigated during the study. This variability is evident even within control groups. To help reach a conclusion on whether a statistically significant finding in a test group is treatment-related account is taken of whether the result in the test group is outside the normal range for untreated animals of the same strain and age. To do this, a number of sources of information are considered, including the standardised effect size, the standard deviations and range of values within test and control groups in the study and, if applicable, data from other studies performed in the same test facility within a small timeframe and under almost identical conditions (Historic Control Data).

Event-specific PCR analysis confirmed the presence of the event DP910521 in both the GM maize grains and diets and excluded the presence of the event in the respective controls.

Both the GM grains and diets were analysed for nutrients, antinutrients and potential contaminants (e.g. selected heavy metals, mycotoxins and pesticides). Balanced diets were formulated based on the specifications for PMI Certified Rodent LabDiet® #5002.

Feed and water were provided ad libitum. In-life procedures and observations and terminal procedures were conducted in accordance with OECD TG 408 (OECD, 2018).

An appropriate range of statistical tests was performed on the results of the study. Detailed description of the methodology and of statistically significant findings identified in rats given a diet containing maize DP910521 is reported in Appendix C.

There were neither test diet-related incidents of mortality nor clinical signs. No test diet-related adverse findings were identified in any of the investigated parameters. A small number of statistically significant findings were noted, but these were not considered adverse effects of treatment for one or more of the following reasons:

- were within the normal variation[23] for the parameter in rats of this age;
- were of small magnitude;
- were identified at only a small number of time intervals with no impact on the overall value;
- exhibited no consistent pattern with related parameters or end-points;
- exhibited no consistency with increasing dose levels.

No gross pathology findings related to the administration of the test diet were observed at necropsy, and the microscopic examination of a wide range of organs and tissues did not identify relevant differences in the incidence or severity of the histopathological findings related to the administration of the test diet compared to the control group.

In this study, an unusually high incidence (6%) of females with mammary gland adenocarcinoma was reported across the groups (one low dose, one high dose and one undosed animal). These tumours are known to occur spontaneously in young Crl:CD(SD) rats. A pathology peer review panel investigated the adenocarcinomas seen in the study and concluded that they were likely to have occurred spontaneously. The GMO Panel concludes, based on the early occurrence of tumours, a single incidence in each group and the report of the pathology peer review panel, that the adenocarcinomas do not represent an effect of exposure to maize DP910521.

The GMO Panel concludes that this 90-day toxicity study is in line with the requirements of Regulation (EU) No 503/2013 and that no adverse effects were observed in rats after feeding diets containing maize DP910521 up to 50% incorporation rate.

3.5.3 | Allergenicity

The strategies to assess the potential risk of allergenicity focus: (i) on the source of the recombinant protein; (ii) on the potential of the newly expressed protein to induce sensitisation or to elicit allergic reactions in already sensitised persons; and (iii) on whether the transformation may have altered the allergenic properties of the modified plant. Furthermore, the assessment also takes into account potential adjuvant properties of the newly expressed proteins, which is defined as the ability to enhance an allergic reaction.

3.5.3.1 | *Assessment of allergenicity of the newly expressed proteins*

A weight-of-evidence approach was followed, taking into account all of the information obtained on the newly expressed protein, as no single piece of information or experimental method yielded sufficient evidence to predict allergenicity (Codex Alimentarius, 2009; EFSA GMO Panel, 2011a, 2017a; Regulation (EU) No 503/2013).

The *cry1B.34*, *pat* and *pmi* genes originate from *B. thuringiensis*, *S. viridochromogenes* and *E. coli*, respectively, none of which are considered common allergenic sources. The safety of the PAT and PMI proteins have been previously assessed by the GMO Panel and no safety concerns were identified (EFSA GMO Panel, 2012, 2017b, 2024).

Updated bioinformatic analyses of the amino acid sequences of the Cry1B.34, PAT and PMI proteins, using the criterion of 35% identity in a sliding window of 80 amino acids, revealed no relevant similarities to known allergens.

The studies on protein stability of the Cry1B.34, PAT and PMI proteins have been described in Section 3.5.1.2. In addition, the GMO Panel did not find any indication that the NEPs Cry1B.34, PAT and PMI at the levels expressed in maize DP910521 might be adjuvants.

[23]Although animals used in a toxicology study are of the same strain, from the same supplier and are closely matched for age and body weight at the start of the study, they exhibit a degree of variability in the parameters investigated during the study. This variability is evident even within control groups. To help reach a conclusion on whether a statistically significant finding in a test group is treatment-related account is taken of whether the result in the test group is outside the normal range for untreated animals of the same strain and age. To do this, a number of sources of information are considered, including the standardised effect size, the standard deviations and range of values within test and control groups in the study and, if applicable, data from other studies performed in the same test facility within a small timeframe and under almost identical conditions (Historic Control Data)

Furthermore, the applicant provided information on the safety of the Cry1B.34, PAT and PMI proteins regarding their potential hazard to cause a coeliac disease response.[24] For such assessment, the applicant followed the principles described in the EFSA GMO Panel Guidance document (EFSA GMO Panel, 2017a). The assessment of the Cry1B.34 protein identified no perfect or relevant partial matches with known coeliac disease peptide sequences. The assessment of the PAT and PMI proteins revealed partial matches containing the Q/E-X1-P-X2 motif and required further investigations. These partial matches have been previously assessed by the EFSA GMO Panel (e.g. EFSA GMO Panel, 2024). Briefly, based on additional considerations on the position and nature of amino acids flanking the motif, such as the presence of two consecutive prolines and the charge and size of adjacent amino acids (EFSA GMO Panel, 2017a), the relevant peptides containing the motif do not raise concern as they fail to mimic gluten sequences. Therefore, no indications of safety concerns were identified by the GMO Panel.

In the context of this application, the GMO Panel considers that there are no indications that the newly expressed Cry1B.34, PAT and/or PMI proteins in maize DP910521 may be allergenic.

3.5.3.2 | *Assessment of allergenicity of the whole GM plant or crop*

The GMO Panel regularly reviews the available publications on food allergy to maize products. However, to date, maize is not considered a common allergenic food[25] (OECD, 2002). Therefore, the GMO Panel does not request experimental data to analyse the allergen repertoire of GM maize.

In the context of this application and considering the data from the molecular characterisation, the compositional analysis and the assessment of the newly expressed proteins (see Sections 3.3, 3.4 and 3.5), the GMO Panel identifies no indications of potentially increased allergenicity of food and feed derived from GM maize DP910521 with respect to that derived from the conventional counterpart and from the non-GM reference varieties tested.

3.5.4 | Dietary exposure assessment to new constituents

In line with Regulation (EU) No 503/2013, the applicant provided dietary exposure estimates to Cry1B.34, PAT and PMI proteins newly expressed in DP910521 maize. Dietary exposure was estimated based on protein expression levels reported in this application for DP910521 maize treated with glufosinate, the currently available consumption data and feed practices, the foods and feeds currently available on the market and the described processing conditions.

For the purpose of estimating dietary exposure, the levels of the newly expressed proteins in DP910521 maize grains, forage and pollen were derived from material harvested in a field trial across six locations in the United States and Canada during the 2020 growing season (Table 1, Section 3.3.4).

3.5.4.1 | *Human dietary exposure*

Chronic and acute estimations of dietary exposure to Cry1B.34, PAT and PMI proteins newly expressed in DP910521 maize were provided. The applicant followed the methodology described in the EFSA Statement 'Human dietary exposure assessment to newly expressed protein in GM foods' to anticipate human dietary exposure making use of summary statistics of consumption (EFSA, 2019a).

Human dietary exposure was estimated across European countries on different population groups: young population (infants, toddlers, 'other children'), adolescents, adult population (adults, elderly and very elderly) and special populations (pregnant and lactating women). Since no specific consumption data were available on commodities containing, consisting of, or obtained from DP910521 maize grains, a conservative scenario with 100% replacement of conventional maize by the GM DP910521 maize was considered. Consumption figures for all relevant commodities (e.g. corn flakes, sweetcorn, popcorn, etc.) were retrieved from the EFSA Comprehensive European Food Consumption Database (EFSA consumption database).[26] Corn oil, corn starch and corn syrup were excluded from the assessment since no proteins are expected to be present in these commodities.

Mean protein expression values on fresh weight basis are considered as the most adequate to estimate human dietary exposure (both acute and chronic) when working with raw primary commodities that are commonly consumed as processed blended commodities (EFSA, 2019a). Different recipes and factors were considered to estimate the amount of maize in the consumed commodities before assigning newly expressed protein levels to the relevant commodities.[27] No losses in

[24]Technical dossier Section 1.5, additional information 30/06/2022.

[25]Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

[26]https://www.efsa.europa.eu/en/applications/gmo/tools. From version updated in March 2022.

[27]Example: 100 g of maize bread is made with ~74 g of maize flour, and a reverse yield factor of 1.22 from the conversion of maize grains into flour is used. This results in ~24.6 μg of Cry1B.34 per gram of maize bread as compared to the 27 μg/g reported as mean concentration in the maize grains.

the newly expressed proteins during processing were considered, except for the above-mentioned commodities excluded from the exposure estimations.

The highest anticipated acute dietary exposure (highly exposed population) was in the age class 'Other children' with estimates up to 410 μg/kg bw per day for Cry1B.34 protein, 198 μg/kg bw per day for PAT protein and 128 μg/kg bw per day for PMI protein. The main contributor to the exposure in the dietary survey with the highest estimates would be corn grains.

The highest anticipated chronic dietary exposure (highly exposed population) was in the age class 'Infants' with estimates up to 220 μg/kg bw per day for Cry1B.34 protein, 106 μg/kg bw per day for PAT protein and 69 μg/kg bw per day for PMI protein. The main contributor to the exposure in the dietary survey with the highest estimates would be corn flakes.

An ad hoc dietary exposure scenario was provided for consumers of pollen supplements under the assumption that these supplements might be made of pollen from maize DP910521. Consumption data on pollen supplements are available for few consumers across seven different European countries.[26] The low number of consumers available adds uncertainty to the exposure estimations which should be interpreted with care, and only allows the estimation of dietary exposure for average consumers. The highest mean acute dietary exposure would be between 0.17 μg/kg bw per day for Cry1B.34 protein and 47 μg/kg bw per day for PAT protein, in the elderly population. Similarly, the highest mean chronic dietary exposure in consumers of pollen supplements would be between 0.12 μg/kg bw per day for Cry1B.34 protein and 32 μg/kg bw per day for PAT protein, also in the elderly population.

3.5.4.2 | *Animal dietary exposure*

Anticipated dietary exposure to Cry1B.34, PAT and PMI proteins in maize DP910521 was estimated across different animal species, assuming the consumption of maize products commonly entering the feed supply chain (i.e. maize grains and forage). A conservative scenario with 100% replacement of conventional maize products by the GM maize DP910521 products was considered.

Mean levels (dry weight) of the newly expressed proteins in grains and forage from maize DP910521 treated with the intended herbicide used for animal dietary exposure are listed in Section 3.3.4, Table 1.

The applicant estimated the dietary exposure to Cry1B.34, PAT and PMI proteins in livestock (i.e. poultry, swine, cattle and sheep), based on estimates for body weights, daily feed intakes and inclusion rates (percentage) of maize grains and forage in rations. Estimated dietary exposure in livestock animals was calculated based on the consumption of maize grain and forage alone or in combination, as reported in Appendix D.

3.5.5 | Nutritional assessment of endogenous constituents

The intended traits of DP910521 maize are resistance against certain lepidopteran insect pests as well as resistance to glufosinate herbicide, with no intention to alter nutritional parameters. However, levels of iron in grain (in plants treated with the intended herbicide) were significantly different from its conventional counterpart and showed a lack of equivalence with the set of non-GM reference varieties (Section 3.4.6). The biological relevance of iron, the role of maize as contributor to its total intake and the magnitude and direction of the observed change were considered during the nutritional assessment.

3.5.5.1 | *Human nutritional assessment*

A small increase of around 5% was observed in the levels of iron in the treated grains of DP910521 maize as compared to its conventional counterpart. Grains and grain-based products are important contributors to iron intake, although in Europe the relevance of maize is much less important than in countries consuming high-maize diets. Additionally, the non-haem iron from plants has lower bioavailability as compared to the haem iron from meat and meat products. In humans, iron is required for oxygen transport, electron transfer, oxidase activities and energy metabolism; dietary reference values (DRVs) for iron were set in 2015 by EFSA (EFSA NDA Panel, 2015), while safe level of intake were recently set (EFSA NDA Panel, 2024). Considering all the evidence, in particular the small magnitude of the increase, the GMO Panel concludes that the levels of iron in DP910521 maize do not raise nutritional concerns.

3.5.5.2 | *Animal nutritional assessment*

Iron is a trace element important in animal nutrition for its physiological function in the metabolism of animals. The small magnitude (5%) of iron increase observed in the treated grains of DP910521 maize, compared to its conventional counterpart, and its low absorption in the GI tract of animals, in particular of pig and poultry in which phytate is present in cereals complexes minerals (Angel et al., 2002; Humer et al., 2015), does not raise concerns. In ruminants, the small magnitude if iron increase does not pose an issue as the ruminal bacteria partially degrade phytate. Furthermore, animal diets are usually balanced with mineral supplements according to the foreseen uses in animal nutrition.

3.5.6 | Post-market monitoring of GM food/feed

Maize DP910521, as described in this application, does not raise any nutritional concern and is as safe as its conventional counterpart and the non-GM reference varieties tested. The GMO Panel concludes that based on the information considered in its safety assessment, a post-market monitoring plan for food and feed is not necessary.

3.5.7 | Conclusions on the food/feed safety assessment

The proteins Cry1B.34, PAT and PMI newly expressed in maize DP910521 do not raise safety concerns for human and animal health. No interactions between the newly expressed proteins relevant for food and feed safety were identified. Moreover, the GMO Panel did not identify indications of safety concerns regarding allergenicity or adjuvanticity related to the presence of the newly expressed proteins in maize DP910521. The GMO Panel finds no evidence that the genetic modification impacts the overall safety of maize DP910521. Based on the outcome of the comparative assessment and the nutritional assessment, the GMO Panel concludes that the consumption of maize DP910521 does not represent any nutritional concern, in the context of the scope of this application. The GMO Panel concludes that maize DP910521, as described in this application, is as safe as the conventional counterpart and the non-GM reference varieties tested, and no post-market monitoring of food/feed is considered necessary.

3.6 | Environmental risk assessment and monitoring plan[28]

3.6.1 | Environmental risk assessment

Considering the scope of application GMFF-2021-2473, which excludes cultivation, the environmental risk assessment (ERA) of maize DP910521 mainly takes into account: i) the exposure of microorganisms to recombinant DNA in the gastrointestinal tract of animals fed with GM material and of microorganisms present in environments exposed to faecal material of these animals (manure and faeces); and ii) the accidental release into the environment of GM material, including viable maize DP910521 grains, during transportation and/or processing (EFSA GMO Panel, 2010a).

3.6.1.1 | *Persistence and invasiveness of the GM plant*

Maize is highly domesticated, not winter hardy in colder regions of Europe, and generally unable to survive in the environment without appropriate management. Survival is limited mainly by a combination of low competitiveness, absence of a dormancy phase and susceptibility to plant pathogens, herbivores and cold climate conditions (OECD, 2003), even though occasional feral GM maize plants may occur outside cultivation areas in the EU (e.g. Pascher, 2016). Field observations indicate that maize grains may survive and overwinter in some EU regions, resulting in volunteers in subsequent crops (e.g. Gruber et al., 2008; Palaudelmàs et al., 2009; Pascher, 2016). However, maize volunteers have been shown to grow weakly and flower asynchronously with the maize crop (Palaudelmàs et al., 2009). Thus, the establishment and survival of feral and volunteer maize in the EU is currently limited and transient.

It is unlikely that the intended traits of maize DP910521 will provide a selective advantage to maize plants, except when they are exposed to glufosinate-containing herbicides or infested by insect pests that are susceptible to the Cry1B.34 protein. However, if this were to occur, this fitness advantage will not allow the GM plant to overcome other biological and abiotic factors limiting plant's persistence and invasiveness. Therefore, the presence of the intended traits will not affect the persistence and invasiveness of the GM plant.

In conclusion, the GMO Panel considers it is very unlikely that maize DP910521 will differ from conventional maize hybrid varieties in their ability to survive until subsequent seasons, or to establish occasional feral plants under European environmental conditions in case of accidental release into the environment of viable maize DP910521 grains.

3.6.1.2 | *Potential for gene transfer*

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either through horizontal gene transfer (HGT) of DNA or through vertical gene flow via cross-pollination from feral plants originating from spilled grains.

Plant-to-microorganism gene transfer

Genomic DNA can be a component of food and feed products derived from maize. It is well documented that such DNA becomes substantially degraded during processing and digestion in the human or animal gastrointestinal tract. However,

[28]Dossier: Part II – Sections 5 and Environmental Monitoring Plan.

bacteria in the digestive tract of humans and animals, and in other environments, may be exposed to fragments of DNA, including the recombinant fraction of such DNA.

Current scientific knowledge of recombination processes in bacteria suggests that horizontal transfer of non-mobile, chromosomally located DNA fragments between unrelated organisms (such as from plants to bacteria) is not likely to occur at detectable frequencies under natural conditions (EFSA, 2009).

Homologous recombination is known to facilitate horizontal transfer of non-mobile, chromosomal DNA fragments to bacterial genomes. This requires the presence of at least two stretches of DNA sequences that are similar in the recombining DNA molecules. In the case of sequence identity with the transgene itself, recombination would result in gene replacement. In the case of identity with two or more regions flanking recombinant DNA, recombination could result in the insertion of additional DNA sequences in bacteria and thus confer the potential for new properties.

In addition to homology-based recombination processes, at a lower transformation rate, the non-homologous end-joining and microhomology-mediated end joining are theoretically possible (EFSA, 2009; Hülter & Wackernagel, 2008). Independently of the transfer mechanism, the GMO Panel did not identify a selective advantage that a theoretical HGT would provide to bacterial recipients in the environment.

Bioinformatic analyses of maize DP910521 revealed that the genetic elements encoding for PAT and Cry1B.34 proteins were plant codon-optimised and did not provide sufficient sequence identity to bacterial DNA. Alignments were detected with the *pmi* coding sequence from *E. coli*. No paired alignments, and thus, no potential to facilitate double HR were identified. Gene replacements of *pmi* sequence on natural *E. coli* might potentially occur in the main receiving environments, i.e. the gastrointestinal tract, but this would not confer any new trait or selective advantage to bacterial recipients.

In summary, there is no indication for an increased likelihood of horizontal transfer of DNA from maize DP910521 to bacteria. Given the nature of the recombinant DNA, the GMO Panel identifies no safety concern linked to an unlikely but theoretically possible HGT.

Plant-to-plant gene transfer

The potential for occasional feral maize DP910521 plants originating from grain import spills to transfer recombinant DNA to sexually compatible plants and the environmental consequences of this transfer were considered.

For plant-to-plant gene transfer to occur, imported GM maize grains need to germinate and develop into plants in areas containing sympatric wild relatives and/or cultivated maize with synchronous flowering and environmental conditions favouring cross-pollination.

Maize is an annual predominantly cross-pollinating crop. Cross-fertilisation occurs mainly by wind (OECD, 2003). Vertical gene transfer from maize is limited to *Zea* species. Wild relatives of maize outside cultivation are not known/reported in Europe (Eastham & Sweet, 2002; EFSA, 2016, 2022; OECD, 2003; Trtikova et al., 2017). Therefore, potential vertical gene transfer is restricted to maize and weedy *Zea* species, such as teosintes, and/or maize-teosinte hybrids, occurring in cultivated areas (EFSA, 2016, 2022; Le Corre et al., 2020; Trtikova et al., 2017).

The potential of spilled maize grains to establish, grow and produce pollen is extremely low and transient (see Section 3.6.1.1). Therefore, the likelihood/frequency of cross-pollination between occasional feral GM maize plants resulting from grain spillage, and weedy or cultivated *Zea* plants is considered extremely low (EFSA, 2016, 2022). Even if cross-pollination would occur, the GMO Panel is of the opinion that environmental effects as a consequence of the spread of genes from occasional feral GM maize plants in Europe will not differ from that of conventional maize varieties for the reasons given in Section 3.6.1.1.

3.6.1.3 | *Interactions of the GM plant with target organisms*

Taking the scope of application GMFF-2021-2473 into account (no cultivation), potential interactions of occasional feral maize DP910521 plants arising from grain import spills with the target organisms are not considered a relevant issue.

3.6.1.4 | *Interactions of the GM plant with non-target organisms*

The GMO Panel evaluated the potential hazards of the NEPs and considered that the environmental exposure of non-target organisms to spilled GM maize material or occasional feral GM maize plants arising from spilled maize DP910521 grains will be limited. Additionally, ingested proteins are typically degraded before entering the environment through faecal material of animals fed with GM maize (Harmon & Swanson, 2020; Miner-Williams et al., 2014; Mok & Urschel, 2020; Santos-Hernández et al., 2018; van Bruchem et al., 1985), and the data provided for the assessment of NEP stability (see Section 3.5.1.2.3) supports that also the NEPs will be degraded. Given the limited environmental exposure, the GMO Panel considers that potential interactions of maize DP910521 with non-target organisms do not raise any environmental safety concern.

3.6.1.5 | *Interactions with abiotic environment and biogeochemical cycles*

The GMO Panel evaluated the potential hazards of the NEPs and considered that the environmental exposure to spilled GM maize material or occasional feral GM maize plants arising from spilled maize DP910521 grains will be limited. Additionally, ingested proteins are typically degraded before entering the environment through faecal material of

animals fed with GM maize (Harmon & Swanson, 2020; Miner-Williams et al., 2014; Mok & Urschel, 2020; Santos-Hernández et al., 2018; van Bruchem et al., 1985), and the data provided for the assessment of NEP stability (see Section 3.5.1.2.3) support that also the NEPs will be degraded. Given the limited environmental exposure, the GMO Panel considers that potential interactions of maize DP910521 with the abiotic environment and biogeochemical cycles do not raise any environmental safety concern.

3.6.2 | Post-market environmental monitoring

The objectives of a post-market environmental monitoring (PMEM) plan, according to Annex VII of Directive 2001/18/EC, are: (i) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the ERA are correct; and (ii) to identify the occurrence of adverse effects of the GMO, or its use, on human health or the environment that were not anticipated in the ERA.

Monitoring is related to risk management, and thus, a final adoption of the PMEM plan falls outside the mandate of EFSA. However, the GMO Panel gives its opinion on the scientific rationale of the PMEM plan provided by the applicant (EFSA GMO Panel, 2011b).

As the ERA did not identify potential adverse environmental effects from maize DP910521, no case-specific monitoring is required.

The PMEM plan proposed by the applicant for maize DP910521 includes: (1) the description of a monitoring approach involving operators (federations involved in import and processing), reporting to the applicant, via a centralised system, any observed adverse effect(s) of GMOs on human health and the environment; (2) a coordinating system established by CropLife Europe for the collection of information recorded by the various operators; and (3) the review of relevant scientific publications retrieved from literature searches (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a PMEM report on an annual basis and a final report at the end of the authorisation period.

The GMO Panel considers that the scope of the PMEM plan provided by the applicant is consistent with the intended uses of maize DP910521. The GMO panel agrees with the reporting intervals proposed by the applicant in its PMEM plan.

3.6.2.1 | *Conclusion of the environmental risk assessment and monitoring plan*

The GMO Panel concludes that it is unlikely that maize DP910521 would differ from conventional maize varieties in its ability to persist under European environmental conditions. Considering the scope of application GMFF-2021-2473, interactions of occasional feral maize DP910521 plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of HGT from maize DP910521 to bacteria does not indicate a safety concern. Therefore, considering the introduced traits, the outcome of the agronomic and phenotypic analysis, and the routes and levels of exposure, the GMO Panel concludes that maize DP910521 would not raise safety concerns in the event of accidental release of GM material, including viable GM maize grains, into the environment.

The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of maize DP910521.

4 | OVERALL CONCLUSIONS

The GMO Panel was asked to carry out a scientific assessment of maize DP910521 for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003.

The molecular characterisation data establish that maize DP910521 contains a single insert consisting of one copy of the *cry1B.34*, *mo-pat* and *pmi* expression cassettes. Bioinformatic analyses of the sequences encoding the newly expressed proteins and other ORFs within the insert or spanning the junctions between the insert and genomic DNA do not raise any safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed over several generations. The methodology used to quantify the levels of Cry1B.34, PAT and PMI proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant- and microbe-produced Cry1B.34 protein, indicate that these two proteins are equivalent, and the microbial-derived protein can be used in the safety studies.

Considering the selection of test materials, the field trial sites and the associated management practices and the agronomic–phenotypic characterisation as an indicator of the overall field trial quality, the GMO panel concludes that the field trials are appropriate to support the comparative analysis. None of the identified differences in the agronomic/phenotypic and compositional characteristics tested between maize DP910521 and its conventional counterpart needs further assessment, except for the levels of iron in grains, which do not raise safety and nutritional concerns. The GMO panel does not identify safety concerns regarding the toxicity and allergenicity of Cry1B.34, PAT and PMI proteins as expressed in maize DP910521, and finds no evidence that the genetic modification impacts the overall allergenicity of maize DP910521. In the context of this application, the consumption of food and feed from maize DP910521 does not represent a nutritional concern in humans and animals. The GMO panel concludes that maize DP910521 is as safe as the conventional counterpart and non-GM maize reference varieties tested, and no post-market monitoring of food/feed is considered necessary.

In the case of accidental release of maize DP910521 material into the environment, this would not raise environmental safety concerns. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of maize DP910521. Based on the relevant publications identified through the literature searches, the GMO panel does not identify any safety issues pertaining to the uses of maize DP910521.

The GMO panel concludes that maize DP910521 is as safe as its conventional counterpart and the tested non-GM maize reference varieties with respect to potential effects on human and animal health and the environment.

5 | DOCUMENTATION AS PROVIDED TO EFSA

- Letter from the Competent Authority of The Netherlands received on 28th June 2022 concerning a request for authorization of the placing on the market of genetically modified maize DP910521, submitted in accordance with Regulation (EC) No 1829/2003 by Corteva Agriscience LLC (EFSA Ref. GMFF-2021-2473; EFSA-Q-2022-00409).
- The application was made valid on 21 December 2022.
- Additional Information (1) was requested on 22 December 2022.
- Additional information (1) was received on 22 February 2023 partial; 8 April complete.
- Additional information (2) was requested on 3 April 2023.
- Additional information (2) was received on 3 June 2023.
- Additional information (3) was requested on 20 June 2023.
- Additional information (3) was received on 4 August 2023.
- Additional information (4) was requested on 17 August 2023.
- Additional information (4) was received on 18 October 2023.
- Additional information (5) was requested on 3 November 2023.
- Additional information (5) was received on 15 January 2024 partial; 24 January 2024 complete.
- Additional information (6) was requested on 21 December 2023.
- Additional information (6) was received on 23 January 2024.
- Additional information (7) was requested on 2 February 2024.
- Additional information (7) was received on 27 February 2024.
- Additional information (8) was requested on 29 February 2024.
- Additional information (8) was received on 2 April 2024.
- Additional information (9) was requested on 10 April 2024.
- Additional information (9) was received on 2 May 2024.

ABBREVIATIONS

ADF	acid detergent fibre
bp	base pair
bw	body weight
CaMV	cauliflower mosaic virus
CRISPR	clustered regularly interspaced short palindromic repeats
dw	dry weight
ELISA	enzyme-linked immunosorbent assay
ERA	environmental risk assessment
fw	fresh weight
GLP	good laboratory practice
GM	genetically modified
GMO	genetically modified organism
GMO Panel	EFSA Panel on Genetically Modified Organisms
HDR	homology-directed repair
HGT	horizontal gene transfer
HR	homologous recombination
JSA	junction sequence analysis
LOQ	limit of quantification
MS	mass spectrometry
NDF	neutral detergent fibre
NEP	newly expressed protein
NGS	next-generation sequencing
OECD	Organisation for Economic Co-operation and Development
ORFs	open reading frames
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PMEM	post-market environmental monitoring
PMI	phosphomannose isomerase

SbS	Southern-by-Sequencing
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SES	standardised effect size
SSI	site-specific integration
TDF	total dietary fibre
TDI	total daily intake
UTR	untranslated region

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CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

Competent Authority of The Netherlands

QUESTION NUMBER

EFSA-Q-2022-00409

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Additional studies

List of additional studies performed by or on behalf of the applicant with regard to the evaluation of the safety of maize DP910521 for humans, animals or the environment.

Study identification	Title
PHI-2021-014/050	(2022) Nutritional Equivalency Study of Maize Grain Containing Event DP-91Ø521-2 – Poultry Feeding Study
PHI-2021-080	(2021) Evaluation of Germination and Viability of Maize Line Containing Event DP-91Ø521-2

APPENDIX B

List of relevant publications identified by the applicant through literature searches (January 2012–November 2023)

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APPENDIX C

Statistical analysis and statistically significant findings in the 28-day toxicity study in mice and in the 90-day toxicity study in rats

C.1 | Statistical analysis of the 28-day toxicity study on the *E. coli*-produced Cry1B.34 protein in mice

The following endpoints were statistically analysed: mortality, clinical signs, body weights, body weight gains, food consumption, food utilisation, functional observational battery, motor activity, ophthalmology, clinical pathology parameters (haematology and clinical chemistry), organ weights, and macroscopic and microscopic examinations. For all continuous endpoints, mean, standard deviation in terms of the standardised effect sizes (SES) of each dose group for each sex, variable, and period of time interval were reported.

The main statistical analysis compared each of the two test diet groups (low and high protein group) separately with the Basal Diet Control group. The analysis was performed for male and female mice separately. Continuous endpoints were analysed with a linear model (factor: diet group); for endpoints measured on a discrete scale, the comparisons were performed with Wilcoxon rank-sum test. For all other ordinal (with fewer than three levels) and nominal (binary) endpoints, the comparison was conducted using Fisher's exact test. Ranges from historical control data were provided for the assessment of statistically significant differences between the test and the control diet group. Missing data were considered by the GMO Panel and found not to have an impact on the results (Tables C.1 and C.2).

TABLE C.1 Statistically significant findings in the 28-day toxicity study on *E.coli*-produced Cry1B.34 protein in mice.

Statistically significant parameter/endpoint	Finding	GMO panel interpretation
Body weight gain and food utilisation	Increased in top dose males days 8–15 (1 g vs. 0 mg in controls) days 22–30 (+0.7 g vs. −0.2 g in controls). Related increases in food utilisation	Small magnitude. Within normal variation. No impact on terminal body weights. Not an adverse effect of treatment
FoB, grooming	Increased in top dose female group	Within normal variation, all values within the control range. Not an adverse effect of treatment
FoB, ambulatory count (interval 1)	Increased (80%) in top dose male group	No significant change in counts over entire observation period. Within normal variation. Not an adverse effect of treatment
FoB, motor activity count (interval 1 & overall)	Increased (40%–50%) in top dose male group	Within normal variation, higher pre-test values (15%–30%). Not an adverse effect of treatment
Mean cell volume	Increased (3%) in low dose male group	Not adverse in isolation, no changes in haematocrit or haemoglobin. Small magnitude. Not seen at top dose. Within normal variation. Not an adverse effect of treatment
ALT	Reduced (31%) in low dose female group	Not adverse in isolation, no changes in other liver parameters. Small magnitude. Not seen at top dose. Within normal variation. Not an adverse effect of treatment
ALP	Increased (25%) in top dose male group	Not adverse in isolation, no changes in other liver parameters. Small magnitude. Within normal variation, driven by one animal (not significant when this animal is treated as an outlier). Not an adverse effect of treatment
Total protein and albumin	Increased (5%–6%) in low dose male group	Small magnitude. Not seen at top dose. Within normal variation. Not an adverse effect of treatment
Total bile acid	Decreased (50%) in low dose male group	Not adverse in isolation. Not seen at top dose. Within normal variation. Not an adverse effect of treatment
Adrenal weight (absolute and relative)	Increased (20%) in both female groups	Within normal variation, all top dose group values are within control range. No associated histopathology findings. Not an adverse effect of treatment
Thyroid/parathyroid weight (absolute)	Decreased (14%) in low dose female group	Small magnitude. Not seen at top dose. Within normal variation. No associated histopathology findings. Not an adverse effect of treatment

Note: Where changes are given as percentages (e.g. reduced (30%)) this indicates the magnitude of the change relative to the control value (e.g. 30% means a value of 7 in test group animals vs. 10 in controls).

C.2 | Statistical analysis of the 90-day toxicity study on maize DP910521 in rats

The following endpoints were statistically analysed: body weights, body weight changes, food consumption, clinical pathology values (as applicable), absolute and relative organ weights, functional observational battery (FOB) data, locomotor activity and histopathological data. For all continuous endpoints, mean, standard deviation in terms of the standardised

effect sizes (SES) of each dose group for each sex, variable and period or time interval were reported. The main statistical analysis compared rats consuming the low-and high-dose test diets (at 17% and 30% inclusion level, respectively) with those consuming the control diet. For continuous parameters, a linear mixed model was applied to data for individual animals for the two sexes combined (fixed effects: diet, sex and sex-by-diet interaction; random effects: block and cage) for each variable and period or time interval. Test-control comparisons were done both across sexes and separately for males and females; in case a significant sex-by-diet interaction was identified, only the sex-specific results were considered for the assessment. The model was modified as needed for the analysis of sex-specific endpoints and cage-level data (food consumption and food efficiency). Ordinal multi-category data were analysed using a Cochran–Mantel–Haenszel test. Binomial category data and unordered multi-category data were analysed by Fisher's exact probabilities test. Missing data were considered by the GMO Panel and found not to have an impact on the results.

TABLE C.2 Statistically significant findings in the 90-day toxicity study on maize DP910521 in rats.

Statistically significant parameter/endpoint	Finding	GMO panel interpretation
Body weight gain and food utilisation	Increased (8%) at low dose in both sexes combined (d1–91). Increased (80%) at top dose in both sexes combined (d78–85)	Low magnitude. Within normal variation. No effect on terminal body weight. No adverse effect of the treatment
Hind limb grip strength	Increased (12%) at top dose in both sexes combined	Low magnitude. Within normal variation. No adverse effect of the treatment
FoB, movement duration in interval 5	Decreased (30%) in low dose female group	No significant change over whole measurement period. Not observed in the high dose animals. No adverse effect of the treatment
Platelet count	Reduced (8%) in the low dose group, sexes combined	Low magnitude. Not observed at high dose animals
APTT	Decreased (8%) in low dose animals, sexes combined	Small magnitude. Not seen at the high dose. No adverse effect of the treatment
BUN	Increased (6%) in low dose animals, sexes combined	Small magnitude. Not observed at the high dose. No adverse effect of the treatment
Creatinine	Increased (15%) in low dose animals, sexes combined	Small magnitude. Not observed at the high dose. No adverse effect of the treatment
Cholesterol	Increased (12%) in high dose animals, sexes combined	Small magnitude. Within normal variation. No adverse effect of the treatment
LDL	Increased (35%) in high dose animals, sexes combined	Mean values influenced by one male and one female with particularly high values. Within normal variation as seen in reference diet values. No adverse effect of the treatment
Total bile acids	Decreased (45%) in low dose males	Decrease is not adverse in isolation. Not observed at high dose. No adverse effect of the treatment
Heart weight (relative to body weight)	Decreased (5%) in top dose animals, sexes combined	Small magnitude. Within normal variation. No associated histopathology findings. No adverse effect of the treatment
Thymus weight (Absolute and relative to brain and body weight)	Increased at the top dose (17%)	Low magnitude. No associated histopathology or white blood cell findings. Within normal variation. No adverse effect of the treatment

Notes. Where changes are given as percentages (e.g. reduced (30%)) this indicates the magnitude of the change relative to the control value (e.g. 30% means a value of 7 in test group animals versus 10 in controls).

APPENDIX D

Animal dietary exposure

TABLE D.1 Dietary exposure to Cry1B.34 protein (mg/kg bw per day) in livestock, based on the consumption of maize grains and forage.

	BW (kg)	TDI feed (kg DM/animal)	IR (%)		Dietary exposure (mg/kg bw per day)		
			Grain (G)	Forage (F)	G	F	G+F
Broiler	1.7	0.12	70	NA	1.6	NA	NA
Layer	1.9	0.13	70	10	1.5	2.7	4.2
Turkey	7	0.50	50	NA	1.1	NA	NA
Breeding pigs	260	6	70	20	0.52	1.8	2.3
Finishing pigs	100	3	70	NA	0.67	NA	NA
Beef cattle[a]	500	12	80	80	0.61	7.5	8.1
Dairy cattle	650	25	30	60	0.37	9.0	9.4
Ram/ewe	75	2.5	30	NA	0.32	NA	NA
Lamb	40	1.7	30	30	0.41	5.0	5.4

Note: NA indicates that a forage inclusion rate was not provided in the reference and therefore no exposure calculations were done.
[a]The inclusion rate for beef cattle would be 160% of the diet, resulting the DDE to each protein an overestimation.

TABLE D.2 Dietary exposure to PAT protein (mg/kg bw per day) in livestock, based on the consumption of maize grain and forage.

	BW (kg)	TDI feed (kg DM/animal)	IR (%)		Dietary exposure (mg/kg bw per day)		
			Grain (G)	Forage (F)	G	F	G+F
Broiler	1.7	0.12	70	NA	0.79	NA	NA
Layer	1.9	0.13	70	10	0.77	0.82	1.86
Turkey	7	0.50	50	NA	0.57	NA	NA
Breeding pigs	260	6	70	20	0.26	0.55	0.81
Finishing pigs	100	3	70	NA	0.34	NA	NA
Beef cattle[a]	500	12	80	80	0.31	2.3	2.6
Dairy cattle	650	25	30	60	0.18	2.8	3
Ram/ewe	75	2.5	30	NA	0.16	NA	NA
Lamb	40	1.7	30	30	0.20	1.5	1.7

Note: NA indicates that a forage inclusion rate was not provided in the reference and therefore no exposure calculations were done.
[a]The inclusion rate for beef cattle would be 160% of the diet, resulting the DDE to each protein an overestimation.

TABLE D.3 Dietary exposure to PMI protein (mg/kg bw per day) in livestock, based on the consumption of maize grain and forage.

	BW (kg)	TDI feed (kg DM/animal)	IR (%)		Dietary exposure (mg/kg bw per day)		
			Grain (G)	Forage (F)	G	F	G+F
Broiler	1.7	0.12	70	NA	0.49	NA	NA
Layer	1.9	0.13	70	10	0.48	0.18	0.66
Turkey	7	0.50	50	NA	0.36	NA	NA
Breeding pigs	260	6	70	20	0.16	0.12	0.29
Finishing pigs	100	3	70	NA	0.21	NA	NA
Beef cattle[a]	500	12	80	80	0.19	0.52	0.71
Dairy cattle	650	25	30	60	0.12	0.62	0.74
Ram/ewe	75	2.5	30	NA	0.10	NA	NA
Lamb	40	1.7	30	30	0.13	0.34	0.47

Note: NA indicates that a forage inclusion rate was not provided in the reference and therefore no exposure calculations were done.
[a]The inclusion rate for beef cattle would be 160% of the diet, resulting the DDE to each protein an overestimation.