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SCIENTIFIC OPINION

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Assessment of genetically modified maize DAS1131 (application GMFF-2021-1530)

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

Genetically modified maize DAS1131 was developed to confer resistance to certain susceptible lepidopteran pests, as well as tolerance to glyphosate herbicide, these properties were achieved by introducing the *cry1Da2* and *dgt-28 epsps* expression cassettes. The molecular characterisation data and bioinformatic analyses do not identify issues requiring food/feed safety assessment. None of the identified differences in the agronomic/phenotypic and compositional characteristics tested between maize DAS1131 and its conventional counterpart needs further assessment, except for crude fat in grain which does not raise safety and nutritional concerns. The Panel on Genetically Modified Organisms (GMO Panel) does not identify safety concerns regarding the toxicity and allergenicity of the Cry1Da2 and DGT-28 EPSPS proteins as expressed in maize DAS1131 and finds no evidence that the genetic modification would change the overall allergenicity of maize DAS1131. In the context of this application, the consumption of food and feed from maize DAS1131 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that maize DAS1131 is as safe as the conventional counterpart and the non-GM maize varieties tested, and no post-market monitoring of food/feed is considered necessary. In the case of release of maize DAS1131 into the environment, including viable grains, 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 DAS1131. The GMO Panel concludes that maize DAS1131 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

Cry1da2, DAS1131, DGT-28 EPSPS, genetic engineering, GM, import and processing, maize (*Zea mays*)

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SUMMARY

Following the submission of application GMFF-2021-1530 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.) DAS1131 according to Regulation (EU) No 503/2013. The scope of application GMFF-2021-1530 is for import, processing, and food and feed uses within the European Union (EU) of maize DAS1131, 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 DAS1131 according to the scope of the application GMFF-2021-1530. The GMO Panel conducted the assessment of maize DAS1131 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 DAS1131 contains a single insert consisting of one copy of the *cry1Da2* and *dgt-28 epsps* expression cassettes. The quality of the sequencing methodology and datasets was assessed by the EFSA GMO Panel and is in compliance to the requirements listed in the EFSA Technical Note. Bioinformatic analyses of the sequences encoding the newly expressed proteins (NEPs), the sequences corresponding to open reading frames (ORFs) within the insert or spanning the junctions between the insert and genomic DNA, as well as the flanking regions, 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 Cry1Da2 and DGT-28 EPSPS proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-produced Cry1Da2 and DGT-28 EPSPS proteins, indicate that these proteins are equivalent and the microbe-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 DAS1131 and its conventional counterpart needs further assessment, except for crude fat in grain which does not raise safety and nutritional concerns. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of the Cry1Da2 and DGT-28 EPSPS proteins as expressed in maize DAS1131 and finds no evidence that the genetic modification would change the overall allergenicity of maize DAS1131. In the context of this application, the consumption of food and feed from maize DAS1131 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that maize DAS1131 is as safe as the 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 DAS1131 would not raise safety concerns in the case of release of GM maize material, including viable grains, into the environment. The post-market environmental monitoring (PMEM) plan and reporting intervals are in line with the intended uses of maize DAS1131.

Based on the relevant publication identified through the literature searches, the GMO Panel does not identify any safety issue pertaining to the intended uses of maize DAS1131.

The GMO Panel concludes that maize DAS1131 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.

1 | INTRODUCTION

The scope of the application GMFF-2021-1530 is for food and feed uses, import and processing of maize DAS1131 and does not include cultivation in the European Union (EU). Maize DAS1131 was developed to confer resistance to certain susceptible lepidopteran pests, as well as tolerance to glyphosate herbicide.

1.1 | Background

On 28 June 2022, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands dossier GMFF-2021-1530 for authorisation of maize DAS1131 (Unique Identifier DAS-Ø1131-3), submitted by Corteva Agriscience LLC (hereafter referred to as 'the applicant') according to Regulation (EC) No 1829/2003.[1]

EFSA checked the application for compliance with the relevant requirements of Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013,[2] with the EFSA guidance documents, and, on 9 January 2023, EFSA declared the application valid and informed EU Member States (MS) and the European Commission (EC) and the dossier was published on Open EFSA.[3]

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 six months to issue a scientific opinion on dossier GMFF-2021-1530. Such time limit was extended whenever EFSA and/or the 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 three months to make their opinion known on dossier GMFF-2021-1530 as of date of 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 DAS1131 in the context of its scope as defined in application GMFF-2021-1530.

According to Regulation (EC) No 1829/2003, this scientific opinion is to be seen as the report requested under Articles 6(6) and 18(6) of that Regulation and thus will be part of the EFSA overall opinion in accordance with Articles 6(5) and 18(5). 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.[5]

2 | DATA AND METHODOLOGIES

2.1 | Data

The applicant submitted a confidential and a non-confidential version of the dossier GMFF-2021-1530 following the EFSA requirements as detailed by EFSA (2021a, 2021b).

In accordance with Art. 38 of the Regulation (EC) No 178/2002[6] 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.[7]

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,[8] 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.

[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]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.

[3]https://open.efsa.europa.eu/questions/EFSA-Q-2022-00410.

[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]These particulars are available online at: https://open.efsa.europa.eu/questions/EFSA-Q-2022-00410.

[6]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.

[7]https://open.efsa.europa.eu/questions/EFSA-Q-2022-00410.

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

The GMO Panel based its scientific assessment of maize DAS1131 on the valid application GMFF-2021-1530, additional information provided by the applicant during the risk assessment, relevant scientific comments submitted by EU Member States and relevant peer reviewed scientific publications. As part of this comprehensive information package, the GMO Panel received two additional unpublished studies submitted by the applicant in order to comply with the specific provisions of Regulation (EU) No 503/2013. These additional unpublished studies are 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 GMO Panel, 2010b, 2018; EFSA, 2010, 2014, 2017, 2018, 2019a, 2019b) for the risk assessment of GM plants.

For this application, in the context of the contracts OC/EFSA/GMO/2018/04, OC/EFSA/MESE/2022/03-01-SC17, OC/EFSA/GMO/2020/01, OC/EFSA/GMO/2021/06, EOI/EFSA/SCIENCE/2020/01 – CT 02 GMO and EOI/EFSA/2022/01 – CT NIF 2023 02 the contractors performed preparatory work for the evaluation of the applicant's literature search, the completeness and quality of DNA sequencing information, the bioinformatic analyses on maize DAS1131 and methods applied for the statistical analysis of the 90-day and 28-day toxicity studies.

3 | ASSESSMENT[9]

3.1 | Introduction

Maize DAS1131 was genetically modified to confer resistance to certain susceptible lepidopteran pests through expression of the Cry1Da2 protein, as well as tolerance to glyphosate herbicide, through expression of the DGT-28 EPSPS protein. 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[10]

The GMO Panel assessed the applicant's literature searches on maize DAS1131, which include a scoping review, according to the guidelines given in EFSA (2010, 2019a).

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-2021-1530. Based on the outcome of the scoping review, the GMO Panel agrees that there is limited value of undertaking a systematic review for maize DAS1131 at present.

The GMO Panel considered the overall quality of the performed literature searches acceptable. The literature searches identified one relevant publication on maize DAS1131 from electronic databases. The relevant publication is listed in Appendix B.

The relevant publication identified through the literature searches did not report information pointing to safety issues associated with maize DAS1131 relevant to the scope of this application.

3.3 | Molecular characterisation[11]

3.3.1 | Transformation process and vector constructs

Maize DAS1131 was developed by *Agrobacterium tumefaciens* (also known as *Rhizobium radiobacter*)-mediated transformation. Immature embryos of maize inbred line B104 were co-cultured with a disarmed *A. tumefaciens* strain DAt13192 containing the vector PHP88492. The plasmid PHP88492 used for the transformation contains two expression cassettes between the right and left border of the T-DNA, containing the following genetic elements:

- The *Cry1Da2* expression cassette consists of the promoter, the 5′ untranslated region (UTR) and intron from the maize *ubiquitin gene 1* (*ubiZM1*), a plant codon-optimised chimeric coding sequence containing sequences from the *cry1Da2* gene encoding an insecticidal core toxin and a modified fragment of the *cry1Ab* gene and the terminator region from the maize *ubiZM1* gene.
- The *dgt-28 epsps* expression cassette consists of the promoter, the 5′ UTR and intron from the *ubiZM1* gene, the

[9] https://open.efsa.europa.eu/dossier/GMFF-2021-1530.

[10] Additional information: 3/12/2024.

[11] Additional information: 24/3/2023, 10/6/2023, 6/9/2023, 17/11/2023, 1/7/2024 and 13/1/2025.

codon-optimised coding sequence of the *5-enolpyruvylshikimate-3-phosphate synthase* (*epsps*) gene from *Streptomyces sviceus* fused to a chimeric chloroplast transit peptide, TraP8, from *Brassica napus* and *Brassica rapa*, and the terminator from the maize *ubiZM1* gene.

The T-DNA of PHP88492 also contains attB1 and attB2 recombination sites and two engineered landing pad regions (ELP1 Region 1 and ELP1 Region 2) (Ainley et al., 2018), and four zinc finger nuclease target recognition sites (ZFN) (Ainley et al., 2013).

The vector backbone contains 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 DAS1131 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 by polymerase chain reaction (PCR) followed by Sanger sequencing to determine the size and organisation of the inserted sequences. The approach used is acceptable in terms of coverage and sensitivity. The quality of the sequencing methodology and datasets was assessed by the EFSA GMO Panel and is in compliance to the requirements listed in the EFSA Technical Note (2018).

NGS/JSA of the whole genome indicated that maize DAS1131 contains a single insert, consisting of a single copy of the T-DNA in the same configuration as in the PHP88492 transformation vector. NGS/JSA also indicated the absence of vector backbone sequences.

Sanger sequencing of PCR amplified fragments determined the nucleotide sequence of 12,247 bp of the insert together with 1289 bp of the 5′ and 1433 bp of the 3′ flanking regions. The Sanger analysis revealed that the insert in maize DAS1131 is identical to the T-DNA of PHP88492, except for the deletion of 27 bp of the right border region, and 390 bp of the left border region and an A to G change at bp 1954 in the ubiZM1 promoter.

The possible interruption of known endogenous maize genes by the insertion in DAS1131 was evaluated by bioinformatics 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 DAS1131.

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

Bioinformatics analyses of the amino acid sequence of the newly expressed proteins reveal no significant similarities to toxins and allergens for Cry1Da2 and DGT-28 EPSPS. 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 (DAS1131_299, DAS1131_411, DAS1131_415, DAS1131_458, DAS1131_643 and DAS1131_685) exceeded the allergenicity assessment threshold of 35% identity using an 80 amino acid sliding window approach. However, five of these ORFs (DAS1131_458, DAS1131_685, DAS1131_643, DAS1131_411, DAS1131_415) are predicted on the complementary strand and lack a known promoter and start codons, while the remaining one (DAS1131_299) is predicted in the coding region of DGT-28 EPSPS but in a different reading frame. No significant similarities with toxins were identified for any ORF within the insert and spanning the junctions between the insert and genomic DNA. In conclusion, these analyses indicated that the expression of an ORF showing significant similarities to toxins or allergens in maize DAS1131 is unlikely.

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination (HR), the applicant performed a sequence identity analysis of the inserted DNA in maize DAS1131, which consists of two expression cassettes containing plant codon optimised NEP coding sequences, with microbial DNA. The likelihood and potential consequences of plant -to -bacteria gene transfer are described in Section 3.5.1.2.

3.3.3 | Protein characterisation and equivalence

Maize DAS1131 expresses two new proteins: Cry1Da2 which confers protection against lepidopteran pests, and DGT-28 EPSPS, which confers tolerance to glyphosate herbicide. Given the technical restraints in producing large enough quantities from plants, these proteins were recombinantly produced in microbial expression systems: *Pseudomonas fluorescens* (Cry1Da2) and *Escherichia coli* (DGT-28 EPSPS). A set of biochemical methods was employed to demonstrate the equivalence between the maize and microbial-derived Cry1Da2 and DGT-28 EPSPS. Purified proteins from these two sources were characterised and compared in terms of their biochemical, structural and functional properties.

Cry1Da2 protein characterisation and equivalence

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis showed that the microbe-produced Cry1Da2 protein had the expected molecular weight of ~68 kDa, while the plant-produced Cry1Da2 migrated at two predominant bands of ~66 and 68 kDa. Both plant and microbe-produced Cry1Da2 proteins were comparably immunoreactive to Cry1Da2 protein specific antibodies. Glycosylation detection analysis demonstrated that none of the Cry1Da2 proteins were glycosylated. Amino acid sequence analysis of the plant-derived Cry1Da2 protein by

mass spectrometry (MS) methods showed that the protein matched the deduced sequence as defined by the *cry1da2* gene. These sequence analysis data were consistent with the previously analysed microbe-produced Cry1Da2. In addition, for the upper band of the plant-produced Cry1Da2 protein, the MS data showed that the N-terminal methionine was acetylated. Such a modification is common in eukaryotic proteins (e.g. Polevoda & Sherman, 2000). For the lower band, LC–MS did not detect the 19 N-terminal amino acids of the Cry1Da2 protein, indicating an N-terminal truncation. This truncation was likely due to proteolysis by trypsin-like proteases in planta or during extraction and purification. Functional equivalence was demonstrated by an insect bioassay which showed that plant and microbe-derived Cry1Da2 proteins had comparable insecticidal activity.

DGT-28 EPSPS protein characterisation and equivalence

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis showed that both plant and microbe-produced DGT-28 EPSPS proteins had the expected molecular weight of ~45 kDa and were comparably immunoreactive to DGT-28 EPSPS protein specific antibodies. Glycosylation detection analysis demonstrated that none of the DGT-28 EPSPS proteins were glycosylated.

The DGT-28 EPSPS precursor protein contains a chloroplast transit peptide (CTP) to target expression of the protein in the chloroplast. The CTP is cleaved upon transport into the chloroplast, resulting in the mature form of the protein. Amino acid sequence analysis of the plant-derived DGT-28 EPSPS protein by MS methods showed that the protein matched the deduced sequence as defined by the *dgt-28 epsps* gene. These sequence analysis data were consistent with the previously analysed microbe-produced DGT-28 EPSPS. In addition, the MS and Edman sequencing data indicated the presence of 2 variants of the plant-produced DGT-28 EPSPS: one beginning with AAR[12] and the other with AR,[11] which shows that the CTP was cleaved at two adjacent positions. Functional equivalence was demonstrated by an in vitro assay which showed that plant and microbe-derived DGT-28 EPSPS proteins had comparable enzymatic activity.

The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-produced Cry1Da2 and DGT-28 EPSPS proteins, indicate that these proteins are equivalent and the microbe-derived proteins can be used in the safety studies.

3.3.4 | Information on the expression of the insert

Protein levels of Cry1Da2 and DGT-28 EPSPS were analysed by an enzyme-linked immunosorbent assay (ELISA) in material harvested in a field trial across six locations in US and Canada during the 2020 growing season. Samples analysed 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 grain (BBCH 87–99) from plants treated and not treated with glyphosate. The mean values and standard deviations of protein expression levels in grains ($n = 24$), forage ($n = 24$) and pollen ($n = 24$) of the Cry1Da2 and DGT-28 EPSPS proteins used to estimate human and animal dietary exposure (see Section 3.4.5) are reported in Table 1.

TABLE 1 Mean values ($n = 24$), standard deviation and ranges of newly expressed proteins in grains [ng/mg dry weight (dw) and ng/mg fresh weight (fw)], pollen and forage (ng/mg dw) from maize DAS1131.

	Glyphosate 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)
Grain (BBCH 87–99)				
Cry1Da2	23[a] ± 6.1[b] (12–33)[c]	19 ± 5.1 (10–28)	26 ± 6.7 (13–39)	22 ± 5.6 (11–33)
DGT-28 EPSPS	42 ± 10 (23–65)	35 ± 8.4 (19–55)	47 ± 13 (27–80)	39 ± 11 (23–67)
Forage (BBCH 85)				
Cry1Da2	46 ± 7.3 (21–58)		42 ± 6.3 (25–54)	
DGT-28 EPSPS	51 ± 19 (27–86)		60 ± 21 (25–120)	
Pollen (BBCH 63–65)				
Cry1Da2	84 ± 8.4 (69–110)		94 ± 17 (73–130)	
DGT-28 EPSPS[(d)]	19 ± 7.0 (< LOQ-31)		26 ± 10 (< LOQ-43)	

[a]Mean value.

[b]Standard deviation.

[c]Range.

[d]Some, but not all, sample results were below the Limit of Quantification (LOQ = 22 ng/mg dw); $N = 15$ for DGT-28 EPSPS in pollen not treated, $N = 18$ in pollen treated, respectively. For dietary exposure estimations (see Section 3.5.4) a value equal to half of the LOQ was used to estimate the mean values when samples were reported as below LOQ.

[12]A, alanine, R, asparagine, according to commonly applied one-letter abbreviations for amino acids.

3.3.5 | Inheritance and stability of inserted DNA

Genetic stability of maize DAS1131 insert was assessed by Southern analysis of genomic DNA from five generations (T1, T2, T3, T4, T6) while inheritance pattern was assessed by quantitative polymerase chain reaction (qPCR)-based segregation analysis and phenotypic analysis (resistance to glyphosate) from five generations: three segregating generations (BC1F1 [Entries 1 and 2] and T2) and two non-segregating generations (T4 and T6). The results indicate that all the plants tested retained a single copy of the insert and flanking regions, which were stably inherited in subsequent generations.

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 DAS1131 contains a single insert consisting of one copy of the *Cry1Da2* and the *dgt-28 epsps* expression cassettes. Bioinformatic analyses of the sequences encoding the newly expressed proteins, the sequences corresponding to ORFs within the insert or spanning the junctions between the insert and genomic DNA, as well as the flanking regions, do not raise any safety concerns. The stability of the inserted DNA and of the introduced traits have been confirmed over several generations. The methodology used to quantify the levels of the Cry1Da2 and DGT-28 EPSPS proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-produced Cry1Da2 and DGT-28 EPSPS proteins, indicate that these proteins are equivalent and the microbe-derived proteins can be used in the safety studies.

3.4 | Comparative analysis[13]

3.4.1 | Overview of studies conducted for the comparative analysis

Application GMFF-2021-1530 presents data on agronomic and phenotypic characteristics, as well as on forage and grain composition of maize DAS1131 (Table 2). In addition, the application contains further data on germination characteristics of grain from maize DAS1131 (see Appendix A).

TABLE 2 Main comparative analysis studies to characterise the maize DAS1131 provided in application GMFF-2021-1530.

Study focus	Study details	Comparator	Non-GM reference varieties
Agronomic and phenotypic analysis	Field study, US and Canada, 2020, 12 sites[a]	Maize hybrid B104/PH4257	16[b]
Compositional analysis	Field study, US and Canada, 2020, 8 sites[a]		

Abbreviation: GM, Genetically modified.

[a]Eight field trials were used for both the agronomic/phenotypic characterisation and the compositional analysis and were located in United States (two in Iowa and Illinois, and one each in Nebraska, Pennsylvania and Texas) and one in Ontario, Canada. Four field trials were used only for the agronomic/phenotypic characterisation and were located in United States (one each in Iowa, Indiana, Missouri) and one in Ontario, Canada.

[b]Non-GM hybrid maize with their corresponding comparative relative maturity indicated in brackets were BK5883 (108), P0843 (108), XL5858 (108), P0928 (109), P0993 (109), XL5939 (109), MY09V40 (109), XL5828 (110), P1093 (110), BK6076 (110), 6046 (110), P1151 (111), XL6158 (111), 33T56 (112), MPS2H721 (112), BK6282 (112).

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 DAS1131 exposed to the intended herbicide (treated), maize DAS1131 not exposed to the intended herbicide (not treated), the comparator B104/PH4257 and four non-GM reference varieties.

The agronomic, phenotypic and compositional data were analysed as specified by EFSA GMO Panel (2010b, 2011a, 2011b). This includes, for each of the two treatments of maize DAS1131, 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 commercial reference varieties). The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).[14]

[13]Additional information: 24/3/2023.

[14]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).

3.4.3 | Suitability of selected test materials

3.4.3.1 | *Selection of the test materials*

Inbred line B104 was transformed to obtain DAS1131, which was then crossed with the inbred line PH4257 to produce the hybrid B104/PH4257 used in the comparative analysis. The comparator used in the field trials is the non-GM maize hybrid B104/PH4257, which has similar genetic background as maize DAS1131 (as documented by the pedigree), and is considered to be the conventional counterpart.

Maize DAS1131 and the conventional counterpart, both with a comparative relative maturity (CRM) of 110, which is considered appropriate for growing in environments across US and Canada, where the comparative field trials were conducted.

Commercial non-GM reference varieties with a CRM ranging from 108 to 112 were selected by the applicant and, at each selected site, four reference varieties were tested (see Table 2). On the basis of the provided information 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 DAS1131 and the conventional counterpart used in the 2020 field trials were produced from plants free of diseases, 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 grains were tested for their germination capacity under warm and cold temperature conditions.[15] Germination capacity of the GM maize DAS1131 was compared with the one of its comparators and the results[16] of these studies indicate that the seed germination of maize DAS1131 was not different than that of its comparator.

3.4.3.3 | *Conclusion on suitability*

The GMO Panel concludes that the maize DAS1131, the conventional counterpart and the non-GM hybrid maize reference varieties were properly selected and are of adequate quality. Therefore, the test materials are considered suitable for the comparative analysis.

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 and Canada. The soil and climatic characteristics of the selected fields were diverse,[17] corresponding to optimal, near -optimal and sub-optimal 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 sum of precipitations were provided on a daily basis. Some exceptional weather conditions were reported at seven of the selected sites.[18] 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 selection of the field trial sites for the comparative analysis.

3.4.4.3 | *Management practices*

The field trials included plots containing maize DAS1131, plots with the conventional counterpart and plots with non-GM maize reference varieties, mostly managed according to local agricultural practices. In addition, the field trials included plots containing maize DAS1131 managed following the same agricultural practices, but conventional herbicides were replaced with the intended glyphosate-containing herbicide that was applied at the BBCH 14 growth stage.[19]

[15]Warm temperature condition corresponds to 25°C for 7 days and cold temperature to 10°C for 7 days followed by 5 days at 25°C.

[16]GM hybrid maize and its conventional counterpart showed a mean germination of 98% under warm and cold temperature conditions.

[17]Soil types of the field trials were clay loam, sandy clay loam, sandy loam, silty clay loam, loam, clay and silt loam; soil organic carbon ranged from 1.0% to 2.5%; pH ranged from 5.7 to 8.0; average temperatures and sum of precipitations during the usual crop growing season ranged respectively from 16.8 to 27.6°C and from 98.6 to 777.5 mm.

[18]Wind storm was registered at two field trials in Iowa and one in Pennsylvania and Texas, heavy rain at one field trial in Iowa and in Ontario, hail at one field trial in Illinois and Texas, and extreme heat at one field trial in Ontario.

[19]BBCH scale describes phenological stages (Meier, 2001).

At three field trial sites, unfavourable environmental conditions led to poor emergence,[20] as a consequence, each site has been resown, resulting in a delayed sowing. The GMO Panel considers that the slightly shorter growing cycle is unlikely to affect the overall representativeness of field trial conditions. Therefore, the management practices, including sowing, harvesting and application of plant protection products were considered acceptable 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 of the field trial sites and most of the management practices applied are typical for receiving environments where the tested materials could be grown.

3.4.5 | Agronomic and phenotypic analysis

Eleven agronomic and phenotypic endpoints[21] plus information on abiotic stressors, disease incidence and arthropod damage were collected from the field trial sites (see Table 2). Three endpoints (dropped ears, ear count and lodging) 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 eight endpoints, with the following results:

- For maize DAS1131 (not treated with the intended herbicide) the test of difference identified statistically significant differences with the conventional counterpart for days to maturity and harvest grain moisture. All the endpoints fell under equivalence category I.
- For maize DAS1131 (treated with the intended herbicide) the test of difference identified statistically significant differences with the conventional counterpart for plant height and final stand count. All the endpoints fell under equivalence category I.

3.4.6 | Compositional analysis

Maize DAS1131 forage and grains harvested from eight sites (Table 2) were analysed for 80 constituents (10 in forage and 70 in grains), including those recommended by OECD (OECD, 2003). The statistical analysis was not applied to nine grain constituents because their concentrations in more than half of the samples were below the limit of quantification.[22]

The statistical analysis was applied to a total of 71 constituents (10 in forage[23] and 61 in grains[24]); a summary of the outcome of the test of difference and the test of equivalence is presented in Table 3:

- For maize DAS1131 not treated with the intended herbicide, statistically significant differences with the conventional counterpart were found for 12 endpoints (2 in forage and 10 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 DAS1131 treated with the intended herbicide, statistically significant differences with the conventional counterpart were found for 16 endpoints (1 in forage and 15 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 crude fat in grain which fell under equivalence category III.

[20]Field trials located in Illinois, Indiana and Missouri experienced respectively cool and wet, dry and saturated soil conditions at the first sowing.

[21]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.

[22]Lauric acid (C12:0), myristic acid (C14:0), heptadecenoic acid (C17:1), eicosadienoic acid (C20:2), riboflavin, β-tocopherol, δ-tocopherol, furfural and raffinose.

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

[24]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, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc), vitamins (β-carotene, α-tocopherol, γ-tocopherol, total tocopherols, thiamine, 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 palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), 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), behenic acid (C22:0), lignoceric acid (C24:0)) and other compounds (p-coumaric acid, ferulic acid, inositol, phytic acid, trypsin inhibitor).

TABLE 3 Outcome of the comparative compositional analysis in grain and forage for maize DAS1131. 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	57	12[d]	53	15[d]
	Category III/IV	–	–	–	1[e]
	Not categorised	2[f]	–	2[f]	–
	Total endpoints	71		71	

[a]Comparison between maize DAS1131 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 DAS1131 and its conventional counterpart falling in equivalence category I-II. For forage, not treated crude protein and phosphorous; treated: moisture. For grain, not treated only: total dietary fibre, heptadecanoic acid (C17:0), oleic acid (C18:1), copper, zinc, inositol; treated only: crude fibre, eicosenoic acid (C20:1), behenic acid (C22:0), threonine, iron, pantothenic acid, cystine, calcium, β-carotene, folic acid; both not treated and treated: palmitoleic acid (C16:1), stearic acid (C18:0), linoleic acid (C18:2), manganese.

[e]Endpoint with significant differences between the maize DAS1131 and its conventional counterpart and falling in equivalence category III: crude fat in grains. Estimated means are reported for these endpoints in Table 4.

[f]Endpoints not categorised for equivalence and without significant differences between the maize DAS1131 and its conventional counterpart: ADF (forage, both not treated and treated) and sodium (grain, both not treated and treated).

The GMO Panel assessed all significant differences between maize DAS1131 and its conventional counterpart, taking into account potential impact on plant metabolism and the natural variability observed for the set of non-GM reference varieties. Quantitative results for the endpoint showing significant differences between maize DAS1131 and its conventional counterpart and falling under category III are given in Table 4.

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

		Maize DAS1131[a]		Conventional counterpart	Non-GM reference varieties	
	Endpoint	Not treated	Treated		Mean	Equivalence limits
Grains	Crude fat (% dw)	3.48	3.39*	3.54	3.91	3.45–4.37

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 DAS1131, 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 or II) and light grey (equivalence category III).

DGT-28 EPSPS is an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Herrmann, 1995). The outcome of the comparative assessment of the aromatic amino acids indicated that no further assessment regarding food and feed safety was required.

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 tested between maize DAS1131 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 DAS1131 and the conventional counterpart needs further assessment regarding food and feed safety except for crude fat in grain, which is further assessed in Section 3.5.5.

3.5 | Food/feed safety assessment[25]

3.5.1 | Overview of overarching information for food/feed assessment

3.5.1.1 | *Compositional analysis*

The compositional analysis of maize DAS1131 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*

Two proteins, Cry1Da2 and DGT-28 EPSPS, are newly expressed in maize DAS1131. These proteins have not been previously assessed by the GMO Panel.

3.5.1.2.1 | *Molecular characterisation*

The Cry1Da2 and DGT-28 EPSPS proteins have been extensively characterised in this application. Furthermore, the equivalence between the maize and microorganism-derived proteins was demonstrated (see 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 Cry1Da2 protein is derived from *Bacillus thuringiensis*, an environmentally ubiquitous bacterium known for producing Bt toxins that protect plants by inhibiting insect and nematode growth. However, Cry1Da2, as expressed in this event, is not a naturally occurring gene from *B. thuringiensis*, but rather a designed chimeric construct (see Section 3.3.1).

The DGT-28 EPSPS protein gene source is *Streptomyces sviceus*, a Gram-positive aerobic bacterium commonly found in soil. There are very few species within the genus *Streptomyces* that are considered pathogenic to plants (Bignell et al., 2010) or animals (Kampfer, 2006).

(b) Information on structure, function and mode of action of NEPs

The insecticidal protein Cry1Da2 confers protection against certain susceptible lepidopteran 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 (Hammond et al., 2013; Jurat-Fuentes & Crickmore, 2017; Koch et al., 2015).

The expressed DGT-28 EPSPS protein is targeted to the maize chloroplasts through the TraP8 peptide to provide tolerance to glyphosate herbicide. The mechanism of action of DGT-28 EPSPS, like other EPSPS enzymes, is the conversion of shikimate-3-phosphate and phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate (Griffin et al., 2021).

(c) Information on identity/homology of NEPs to other proteins in conventional sources

The GMO Panel is not aware of any information on identity/homology of Cry1Da2 to other proteins in conventional food and feed sources.

The DGT-28 EPSPS protein belongs to a newly discovered Class IV EPSPS. This class of enzymes have been investigated via crystallography and kinetics analyses (Griffin et al., 2021) The phylogenetic analysis showed that the newly discovered Class IV EPSPS is most closely related to Class I family members, which contains (i) the native enzymes EPSPS from *Glycine max* and *Zea mays* consumed as part of the normal diet by humans and animals and have a history of safe use; and (ii) the 2mEPSPS protein previously assessed by the EFSA GMO Panel (EFSA GMO Panel, 2017, 2020). Structural comparison of full-length DGT-28 EPSPS with EPSPS proteins present in edible crop tissues (e.g. native EPSPS proteins in maize and soybean) or EPSPS previously assessed by the EFSA GMO Panel (e.g. CP4 EPSPS, 2mEPSPS), showed that DGT-28 EPSPS holds the highest overall 3D structure similarity to the class I *E. coli* EPSPS bacterial enzyme, followed closely by the class I native soybean and maize EPSPS.[26] In addition, the active site architecture was demonstrated to be highly conserved between all EPSPS enzymes and the active site amino acid residues involved in substrate binding and catalysis are highly similar between DGT-28 EPSPS and Class I (*E. coli* EPSPS, and native maize and soybean EPSPS) enzymes.[27] The GMO Panel concluded that the DGT-28 EPSPS protein and native maize and soybean EPSPS proteins share both highly conserved regions of tertiary structures involved in substrate binding and catalysis as well as the same mechanism of action.

[25]Additional information: 24/3/2023, 6/9/2023, 17/11/2023, 21/2/2024, 22/2/2024, 30/4/2024, 28/6/2024, 1/7/2024 and 11/10/2024.
[26]Additional information: 15/2/2024, the Root-Mean-Square Deviation values for the full sequences assessed ranged from 0.78 to 1.01.
[27]Additional information: 17/11/2023, the Root-Mean-Square Deviation values for the catalytic pocket assessed ranged from 1.04 to 1.06.

In summary, the GMO Panel acknowledges the functional and structural similarity of the DGT-28 EPSPS protein with both native EPSPS proteins commonly consumed as part of the normal diet by humans and animals, and previously assessed by the EFSA GMO Panel.

(d) Overall conclusion of the history of safe use

The GMO Panel considers the above information not sufficient to duly document the history of safe use for consumption of the Cry1Da2 and DGT-28 EPSPS proteins.

3.5.1.2.3 | *Substrate specificity*

The GMO Panel assessed the substrate specificity of other EPSPS proteins in the past (e.g. EFSA GMO Panel (2022). The mechanism of action of EPSPS proteins is a biochemical reaction involving conversion of shikimate-3-phosphate and phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate. The GMO Panel is not aware of additional information that would change its previous assessments.

3.5.1.2.4 | *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, 2017, 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 that a prominent trait attributed to food allergens and relevant for protein safety is protein stability (Breiteneder & Mills, 2005; Costa et al., 2021; Foo & Müller, 2021; Helm, 2001).

(a) Effect of temperature and pH on NEPs

The applicant provided experimental studies on the effects of temperature on the Cry1Da2 and DGT-28 EPSPS proteins as expressed in maize DAS1131, using a microbial recombinant system. Independent samples of the Cry1Da2 protein were incubated for 30 mins at 25°C, 50°C, 75°C and 95°C followed by a bioassay measuring its functional activity. The study showed that the functional activity of the Cry1Da2 protein was diminished at temperatures of 75°C or above. Similarly, samples of the DGT-28 EPSPS protein were incubated for 30 min at 25°C, 37°C, 50°C and 75°C followed by an enzymatic activity assay. The study showed that the enzymatic activity of the DGT-28 EPSPS protein was lost at temperatures of 50°C or above.

In relation to the effect of pH on the Cry1Da2 and DGT-28 EPSPS proteins, the molecular mass and immunoreactivity of the proteins were unchanged at pH 1.2 and 7.5.

(b) In vitro protein degradation by proteolytic enzymes

The applicant provided independent studies on in vitro protein degradation (i.e. resistance to pepsin in solutions at pH ~ 1.2) of the Cry1Da2 and DGT-28 EPSPS proteins as expressed in maize DAS1131. The integrity of the test Cry1Da2 and DGT-28 EPSPS proteins in samples incubated at various time points was analysed by SDS-PAGE followed by protein staining or by Western blotting.

Samples of the Cry1Da2 protein were degraded by pepsin within 0.5 min of incubation. Two large visible bands of ~60 kDa and ~ 15 kDa disappeared at 1 and 5 min, respectively. Low molecular weight bands ~2–5 kDa were seen for up to 60 min. The applicant provided an additional study, as part of the dossier, where the Cry1Da2 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 the physiological conditions of gastrointestinal digestion (EFSA GMO Panel, 2021). This is in line with Codex Alimentarius which indicated that alternative in vitro digestion protocols may be used, where adequate justification is provided (Codex Alimentarius, 2009).

Samples of the DGT-28 EPSPS protein were degraded by pepsin within 0.5 min of incubation. Low molecular weight bands ~2–5 kDa were seen for up to 60 min. The applicant provided an additional study, as part of the dossier, where the DGT-28 EPSPS 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.

3.5.1.2.5 | *Synergistic or antagonistic interactions*

The potential for a functional interaction among the Cry1Da2 and DGT-28 EPSPS proteins has been assessed with regard to human and animal health. Based on current scientific knowledge on the biological function of the two proteins (Section 3.5.1.2, Table 5), no synergistic or antagonistic interactions between these three proteins which could raise safety concerns for food and feed from maize DAS1131 are expected.

TABLE 5 Intended effects and modes of action of the NEPs in maize DP910521.

Protein	Intended effect and mode of action
Cry1Da2	The Cry1Da2 protein confers resistance to lepidopteran pests by causing disruption of the midgut epithelium via pore formation.
DGT-28 EPSPS	The DGT-28 EPSPS protein confers tolerance to glyphosate-containing herbicides, acting on the shikimic acid pathway for the biosynthesis of aromatic amino acids in bacteria, fungi and plants.

3.5.1.3 | *Effects of processing*

Maize DAS1131 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 genetically modified 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*

Two proteins (Cry1Da2 and DGT-28 EPSPS) are newly expressed in maize DAS1131.

NEP never assessed before

A weight-of-evidence approach was followed by the GMO Panel to assess the toxicological profile of the newly expressed Cry1Da2 and DGT-28 EPSPS proteins, taking into account all of the information relevant for their hazard assessment, including molecular characterisation (Section 3.3), 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), updated bioinformatic analyses for similarity to toxins and in vivo toxicity studies.

3.5.2.1.1 | *Bioinformatic analyses*

Updated bioinformatics analyses of the amino acid sequence of the Cry1da2 and DGT-28 EPSPS proteins revealed no significant similarities to known toxins (Section 3.3.2).

3.5.2.1.2 | In vivo *toxicity studies*

For the assessment of the Cry1Da2 and DGT-28 EPSPS proteins, the applicant provided acute toxicity studies, which are described below.

For the assessment of the Cry1Da2 protein, the applicant provided a 28-day toxicity study, which is described below. In contrast, a 28-day study with the DGT-28 EPSPS protein was not provided by the applicant. The GMO Panel concluded that this study was unnecessary for the assessment of the DGT-28 EPSPS protein based on the (i) molecular characterisation; (ii) information on homology to other proteins in conventional sources; (iii) absence of significant similarities to known toxins; and (iv) lack of stability of the protein.

Acute toxicity studies

An acute toxicity study in Crl:CD1(ICR) mice, administrated the microorganism-produced (*P. fluorescens*) Cry1Da2 protein by gavage at the dose of 5000 mg/kg (bw) showed no adverse effects.

An acute toxicity study in Crl:CD1(ICR) mice, administrated the microorganism-produced (*E. coli*) DGT-28 EPSPS protein by gavage at the dose of 2000 mg/kg (bw) showed no adverse effects.

28-day repeated dose toxicity study with Cry1Da2 protein

The applicant provided a 28-day repeated dose toxicity study in mice with Cry1Da2 protein, 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), approximately 8-week old at the start of dosing were allocated to three groups. Groups were administered diets containing respectively the test substance (Cry1Da2 protein) at targeted nominal

doses of 1000 or 300 mg/kg body weight (bw) per day (high and low Cry1Da2 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 the purity of Cry1Da2 protein was 95%. The amino acid sequence analysis of the *P. fluorescens* – produced Cry1Da2 used in this 28-day toxicity study by mass spectrometry matched the deduced sequence as defined by the *cry1Da2* gene. This protein had the expected molecular weight and immunoreactivity to Cry1Da2 specific antibodies, was not glycosylated and showed functional activity.

In-life procedures and observations and terminal procedures were conducted in accordance to OECD TG 407 (2008), except for satellite animals that were not subjected to some in-life procedure (ophthalmology, functional observational battery, motor activity), clinical chemistry and pathology investigations.

The GMO Panel noted that animals were singly housed. 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 analysis tests indicated that the diets preparations were homogeneous and exhibited acceptable stability. Achieved mean intakes at the low concentration were 257 and 352 mg/kg bw per day in males and females respectively and at the high concentration were 879 and 1170 mg/kg bw per day in males and females respectively.

An appropriate range of statistical tests were 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 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 endpoints.
- 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 *P. fluorescens* – produced Cry1Da2 protein, at nominal dietary exposures up to 1000 mg/kg bw per day.

3.5.2.2 | *Assessment of new constituents other than newly expressed proteins*

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 seed and forage from maize DAS1131. Therefore, no further food and 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 seed and forage from maize DAS1131, except for crude fat in grain. These changes are considered not to represent a toxicological concern, considering the biological role of the affected constituent and the magnitude of the changes, therefore, no further toxicological assessment is needed. Further information on the relevance of these findings is provided in Section 3.5.5 (human and animal nutrition).

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

Based on the outcome of the molecular characterisation, toxicological and comparative analysis assessment, no compositional modifications or indications of possible unintended effects relevant to food and feed safety have been identified for maize DAS1131. Therefore, animal feeding studies with food/feed derived from maize DAS1131 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 DAS1131.

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 DAS1131 grains from plants treated with the intended herbicide (glyphosate) at 50% and 33% of inclusion level (the latter supplemented with 17% of the non-GM comparator maize), the non-GM comparator (inclusion level 50%) and the three reference varieties (BK5883, P0843, 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 EFSA Explanatory statement (EFSA, 2014) and complied with the principles of good

laboratory practice (GLP) with some minor deviations described in the study report, not impacting the study results and interpretation.

The stability of the test and control materials was not analytically verified; however, it was confirmed that the diet was used in accordance to product expiration declared by the diet manufacturer. The GMO Panel considered this 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. The applicant provided information on concentration of the newly expressed proteins Cry1Da2 in the GM diets, further supporting the homogeneity of the formulations.

Event-specific PCR analysis confirmed the presence of the event DAS1131 in both the GM grains and diets and excluded the presence of the event in the respective controls. ELISA analyses also confirmed the presence of the event DAS1131 in the GM maize grains and GM diets.

Both the GM and control grains and diets were analysed for nutrients, antinutrients and potential contaminants. Balanced diets were formulated based on the specifications for PMI Nutrition International, LLC Certified Rodent LabDiet® 5002. Feed and water were provided ad libitum. In-life procedures and observations and terminal procedures were conducted in accordance to OECD TG 408 (2018).

An appropriate range of statistical tests were performed on the results of the study. Detailed description of the methodology and of statistically significant findings identified in rats given diets containing grains/meal derived from maize DAS1131 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[28] 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 endpoints.
- exhibited no consistency with increasing incorporation 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 histopathological findings related to the administration of the test diet compared to the control group.

In this study a single incidence of mammary gland adenocarcinoma was reported in a low dose female exposed to DAS1131. These tumours are known to occur spontaneously in young SD rats. A pathology working group investigated the adenocarcinoma seen in the study with DAS1131 and concluded that it was likely to have occurred spontaneously. The GMO panel concludes, based on the single incidence in low-dose group and the report of the pathology working group, that the adenocarcinoma does not represent an effect of exposure to maize DAS1131.

The GMO Panel concludes that this study is in line with the requirements of Regulation (EU) No 503/2013 and that no treatment related adverse effects were observed in rats after feeding diets containing maize DAS1131 grains at 50% or 33% for 90 days.

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, 2017; Regulation (EU)No 503/2013).

The *cry1Da2* and *dgt-28 epsps* genes originate from *B. thuringiensis* and *S. sviceus*, respectively, none of which are considered common allergenic sources.

[28]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).

Updated bioinformatic analyses of the amino acid sequences of the Cry1Da2 and DGT-28 EPSPS proteins, using the criterion of more than 35% identity in a sliding window of 80 amino acids, revealed no relevant similarities to known allergens.

The studies on protein stability of the Cry1Da2 and DGT-28 EPSPS proteins have been described in Section 3.5.1.2.3. Regarding the DGT-28 EPSPS protein, information on homology to other proteins in conventional sources was considered in the assessment. Moreover, the GMO Panel did not find an indication that the newly expressed Cry1Da2 and DGT-28 EPSPS proteins at the levels expressed in maize DAS1131 might be adjuvants.

Furthermore, the applicant provided information on the safety of the Cry1Da2 and DGT-28 EPSPS proteins regarding their potential hazard to cause a celiac disease response.[23,24,29] For such assessment, the applicant followed the principles described in the EFSA GMO Panel guidance document (EFSA GMO Panel, 2017). The assessment of the DGT-28 EPSPS protein identified no perfect or relevant partial matches with known celiac disease peptide sequences. The assessment of the Cry1Da2 protein revealed two partial matches containing a novel 4 amino acid motif defined by the applicant that resembles the known 4 amino acid motif (Q/E-X1-P-X2) and required further investigations.[30] These partial matches were assessed by the EFSA GMO Panel considering that the motif defined by the applicant still requires confirmation about its relevance for risk assessment. Based on additional considerations on the position and nature of amino acids flanking the motif, such as the charge and size of adjacent amino acids and their likelihood to be digested by gastrointestinal proteases[31] (see also Section 3.5.1.2.3 above; EFSA GMO Panel, 2017), the GMO Panel considers that the relevant peptides containing the potential motif do not raise concern.

In the context of this application, the GMO Panel considers that there are no indications that the newly expressed Cry1Da2 and/or DGT-28 EPSPS proteins in GM maize DAS1131 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. However, maize is not considered a common allergenic food[32] (OECD, 2002). Therefore, the GMO Panel does not request experimental data as a routine basis 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 3.2, 3.3 and 3.4), the GMO Panel identifies no indications of a potentially increased allergenicity of food and feed derived from this GM maize DAS1131 with respect to that derived from the conventional counterpart and 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 the Cry1Da2 and DGT-28 EPSPS proteins newly expressed in maize DAS1131. Dietary exposure was estimated based on protein expression levels reported in this application for maize DAS1131 treated with glyphosate, 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 maize DAS1131 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*

Estimates of chronic and acute dietary exposure to Cry1Da2 and DGT-28 EPSPS proteins newly expressed in maize DAS1131 were provided. The applicant followed the methodology described in the EFSA Statement 'Human dietary exposure assessment to newly expressed proteins 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 DAS1131 maize grains, a conservative scenario with 100% replacement of conventional maize by the GM maize was considered. Consumption figures for all relevant commodities (e.g. corn flakes, sweet corn, popcorn, etc.) were retrieved from the EFSA Comprehensive European Food Consumption Database (EFSA consumption database).[33]

[29]Technical dossier Section 1.5, additional information 30/6/2022.

[30]Additional information 28/6/2024 and https://r4eu.efsa.europa.eu/app/predq.

[31]Technical dossier Section 1.5, additional information 28/6/2024.

[32]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.

[33]https://www.efsa.europa.eu/en/applications/gmo/tools. EFSA consumption database: version 1.0 (updated March 2022).

Corn oil, corn starch and corn syrup were excluded from the assessment since no proteins are expected to be present in these commodities.[34]

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. No losses in the newly expressed proteins during processing were considered, except for the commodities mentioned above.

The highest anticipated acute dietary exposure (highly exposed population) was estimated in the age class 'Other children' with estimates of 334 µg/kg bw per day and 593 µg/kg bw per day for Cry1Da2 and DGT-28 EPSPS proteins, respectively. 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 estimated in the age class 'Infants' with estimates of 180 µg/kg bw per day and 318 µg/kg bw per day for Cry1Da2 and DGT-28 EPSPS proteins, respectively. 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 DAS1131 maize. Consumption data on pollen supplements are available for few consumers across seven different European countries.[31] The consumption data based on a low number of consumers adds uncertainty to the exposure estimations which should be carefully interpreted, and only allows the estimation of dietary exposure for average consumers. The highest mean acute dietary exposure would be between 17.8 µg/kg bw per day for DGT-28 EPSPS and 65.2 µg/kg bw per day for Cry1Da2, in the elderly population. Similarly, the highest mean chronic dietary exposure in consumers of pollen supplements would be between 11.9 µg/kg bw per day for DGT-28 EPSPS and 43.5 µg/kg bw per day for Cry1Da2, also in the elderly population.

3.5.4.2 | *Animal dietary exposure*

Anticipated dietary exposure to Cry1Da2 and DGT-28 EPSPS proteins in maize DAS1131 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 DAS1131 products was considered.

Mean levels (dry weight) of the newly expressed proteins in grains and forage from maize DAS1131 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 Cry1Da2 and DGT-28 EPSPS 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, as provided for the EU by OECD (2013). 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 comparative compositional analysis revealed differences in grain composition between maize DAS1131 and its conventional counterpart that needs further assessment regarding food and feed safety. This refers in particular to the levels of crude fat (see Table 4).

The biological relevance of this component, the role of maize as contributor to its total intake and the magnitude and direction of the observed changes were considered during the nutritional assessment.

3.5.5.1 | *Human nutrition*

The levels of crude fat in grain were approximately ~4% lower in treated maize DAS1131 compared to its conventional counterpart. The EFSA NDA Panel has not defined a lower threshold intake (LTI) or a tolerable upper intake level (UL) for total fat but proposed a Reference Intake range of 20–35% of total dietary energy (E%) (EFSA NDA Panel, 2010). Considering the small magnitude of the decrease and the fact that there are other sources of fat in human diet, the decrease in crude fat in maize DAS1131 does not raise any nutritional concern.

3.5.5.2 | *Animal nutrition*

Animal complete diets are balanced to energy content, i.e. crude fat from feed ingredients are taken into account to meet animal nutritional requirements. Considering the small magnitude of the decrease (4%) as compared to the conventional counterpart, the nutritional impact in feeds is considered negligible.

[34]Example: 100 grams of maize bread are made with approximately 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 ~ 19.9 µg of Cry1Da2 (w.w.) per gram of maize bread as compared to the 22 µg/g (w.w.) reported as mean concentration in the maize grains.

3.5.6 | Post-market monitoring of GM food/feed

Maize DAS1131, 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 newly expressed Cry1Da2 and DGT-28 EPSPS proteins in maize DAS1131 do not raise safety concerns for human and animal health. No interactions between the newly expressed Cry1Da2 and DGT-28 EPSPS 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 Cry1Da2 and DGT-28 EPSPS. The GMO Panel found no evidence that the genetic modification impacts the overall safety of maize DAS1131. Based on the outcome of the comparative assessment and the nutritional assessment, the GMO Panel concludes that the consumption of maize DAS1131 does not represent any nutritional concern, in the context of the scope of this application. The GMO Panel concludes that maize DAS1131, 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

3.6.1 | Environmental risk assessment

Considering the scope of application GMFF-2021-1530, which excludes cultivation, the environmental risk assessment (ERA) of maize DAS1131 mainly takes into account: (1) 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 manure and faeces of these animals; and (2) the accidental release into the environment of GM material, including viable maize DAS1131 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 DAS1131 will provide a selective advantage to maize plants, except when they are exposed to glyphosate-containing herbicides or infested by insect pests that are susceptible to the Cry1Da2 protein. However, if this was to occur this fitness advantage will not allow the GM plant to overcome other biological and abiotic factors (described above) 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 DAS1131 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 DAS1131 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 DAS1131. It is well documented that such DNA becomes substantially degraded during processing and digestion in the human or animal gastrointestinal tract. However, 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 (for further details, see 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 analysis of event DAS1131 revealed that there are no elements providing sufficient similarity to bacterial DNA which would facilitate homologous recombination including the sequences of bacterial origin encoding for Cry1Da2 and DGT-28 EPSPS proteins that were all plant codon-optimised (see Section 3.3.1).

In summary, there is no indication for an increased likelihood of horizontal transfer of DNA from maize DAS1131 to bacteria. Given the nature of the recombinant DNA, the GMO Panel identified no safety concern linked to an unlikely but theoretically possible HGT.

Plant-to-plant gene transfer

The potential for occasional feral maize DAS1131 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; Trtikova et al., 2017; Corre et al., 2020).

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-2021-1530 into account (no cultivation), potential interactions of occasional feral maize DAS1131 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 DAS1131 grains will be limited. Additionally, ingested proteins are typically degraded before entering the environment through manure and faeces of animals fed with GM maize (van 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 protein stability (see Section 3.5.1.2.3) supports that also the NEPs will be degraded. As compared to non-GM modified maize, the GMO Panel considers that potential interactions of maize DAS1131 with non-target organisms do not raise any environmental safety concern.

3.6.1.5 | *Interactions with 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 DAS1131 grains will be limited. Additionally, ingested proteins are typically degraded before entering the environment through manure and faces of animals fed with GM maize (van Bruchem et al., 1985; Miner-Williams et al., 2014; Santos-Hernández et al., 2018; Harmon & Swanson, 2020; Mok & Urschel, 2020), and the data provided for the assessment of protein stability (see section 3.5.1.2.3) supports that also the NEPs will be degraded. Based on the comparative approach, the GMO Panel considers that potential interactions of maize DAS1131 with the 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: (1) 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 (2) 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 DAS1131, no case-specific monitoring is required.

The PMEM plan proposed by the applicant for maize DAS1131 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 for the duration 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 DAS1131. 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 DAS1131 would differ from conventional maize varieties in its ability to persist under European environmental conditions. Considering the scope of application GMFF-2021-1530, interactions of occasional feral maize DAS1131 plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of HGT from maize DAS1131 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 DAS1131 would not raise safety concerns in the event of 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 DAS1131.

4 | OVERALL CONCLUSIONS

The GMO Panel was asked to carry out a scientific assessment of maize DAS1131 for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003.

The molecular characterisation data establish that maize DAS1131 contains a single insert consisting of one copy of the *cry1Da2* and *dgt-28 epsps* expression cassettes. The quality of the sequencing methodology and datasets was assessed by the EFSA GMO Panel and is in compliance with the requirements listed in the EFSA Technical Note. Bioinformatic analyses of the sequences encoding the newly expressed proteins, the sequences corresponding to ORFs within the insert or spanning the junctions between the insert and genomic DNA, as well as the flanking regions, do not raise any safety concerns. The stability of the inserted DNA and of the introduced traits is confirmed over several generations. The methodology used to quantify the levels of the Cry1Da2 and DGT-28 EPSPS proteins is considered adequate. The protein characterisation data comparing the biochemical, structural and functional properties of plant and microbe-produced Cry1Da2 and DGT-28 EPSPS proteins, indicate that these proteins are equivalent and the microbe-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 DAS1131 and its conventional counterpart needed further assessment, except for the levels of crude fat 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 Cry1Da2 and DGT-28 EPSPS proteins as expressed in maize DAS1131, and finds no evidence that the genetic modification would change the overall allergenicity of maize DAS1131. In the context of this application, the consumption of food and feed from maize DAS1131 does not represent a nutritional concern in humans and animals. The GMO Panel concludes that maize DAS1131 is as safe as the 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 DAS1131 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 DAS1131. Based on the relevant publication identified through the literature searches, the GMO Panel does not identify any safety issues pertaining to the uses of maize DAS1131. The GMO Panel concludes that maize DAS1131 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

The documentation is available online at: https://open.efsa.europa.eu/questions/EFSA-Q-2022-00410.

ABBREVIATIONS

3D	three dimensional
ADF	acid detergent fibre
ALT	Alanine Aminotransferase
attB	Bacterial Attachment Sites
BBCH	Biologische Bundesanstalt, Bundessortenamt and CHemical industry
Bp	base pair
Bt toxin	*Bacillus thuringensis* toxin
bw	body weight
CMH	Cochran–Mantel–Haenszel () test
CRM	comparative relative maturity
CRY	crystal proteins
CTP	chloroplast transit peptide
DM	dry matter
dw	dry weight
E%	total dietary energy
ELISA	Enzyme-Linked Immunosorbent Assay
ELP	Engineered Landing Pad
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ERA	environmental risk assessment
FOB	functional observational battery
fw	fresh weight
GLP	good laboratory practice
GM	genetically modified
GMO	genetically modified organisms
HGT	horizontal gene transfer
HR	homologous recombination
IR	(%) percentage of inclusion rate
JSA	junction sequence analysis
LC–MS	liquid chromatography–mass spectrometry
LOQ	limit of quantification
LTI	lower threshold intake
MS	mass spectrometry
NA	not applicable
NDF	neutral detergent fibre
NEP	newly expressed protein
NGS	Next Generation Sequencing
OECD	organisation for Economic Co-operation and Development
ORFs	open reading frames
PCR	polymerase chain reaction
PMEM	post-market environmental monitoring
qPCR	quantitative polymerase chain reaction
SbS	Southern-by-sequencing
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SES	standardised effect sizes
TDF	total dietary fibre
TDI	total daily intake
T-DNA	transfer- deoxyribonucleic acid
TSH	thyroid-stimulating hormone
UL	tolerable upper intake level
UTR	untranslated region
ZFN	zinc finger nuclease

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REQUESTOR

Competent Authority of the Netherlands

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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 DAS1131 for humans, animals or the environment.

Study identification	Title
PHI-2021-014/051	(2022) Nutritional Equivalency Study of Maize Grain Containing Event DAS-Ø1131-3 – Poultry Feeding Study
PHI-2021-210	(2021) Evaluation of Germination and Viability of Maize Line Containing Event DAS-Ø1131-3

APPENDIX B

List of relevant publications identified by the applicant through literature searches (January 2012 – November 2024)

Reference

Griffin, S. L., Chekan, J. R., Lira, J. M., Robinson, A. E., Yerkes, C. N., Siehl, D. L., Wright, T. R., Nair, S. K., & Cicchillo, R. M. (2021). Characterization of a glyphosate-tolerant enzyme from *Streptomyces svecius*: A distinct class of 5-Enolpyruvylshikimate-3-phosphate Synthases

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 *P. fluorescens* - produced Cry1Da2 protein in mice

The following endpoints were statistically analysed: mortality, clinical signs, body weight, body weight gains, food consumption, food utilisation, haematology variables, coagulation variables, clinical chemistry variables, functional observational battery continuous data, functional observational battery categorical data, motor activity data, organ weights, organ weight relative to body weight, organ weight relative to brain weight. 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 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. Missing data were considered by the Panel and found not to have an impact on the results (Table C.1).

TABLE C.1 Statistically significant findings in the 28-day toxicity study on *P. fluorescens* -produced Cry1Da2 protein in mice.

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
Body weight gain and food utilisation	Reduced in low dose males days 8–15 (0.5 vs. 1.4 g in controls). Related decreases in food utilisation.	Within normal variation. No impact on terminal body weights. Not seen at the top dose. Not an adverse effect of treatment.
Urination counts	Decreased (50%) in low dose male group.	Within normal variation, all values within the control range. Not an adverse effect of treatment.
Time to first step	Increased (25%) in top dose female group.	Within normal variation, all values within the control range. Not an adverse effect of treatment.
ALT	Reduced (25%) in low dose male group.	Not adverse in isolation. Control mean influenced by one result, 70% above next highest value. Within normal variation. Not an adverse effect of treatment.
Bilirubin	Increased (46%) in low dose male group.	Not present at the top dose or in females. Within normal variation. Not an adverse effect of treatment.
Calcium	Increased (3%) in low dose female group.	Small magnitude. Not seen at top dose. Within normal variation. Not an adverse effect of treatment.
Potassium	Decreased (10%) in top dose male group.	Small magnitude. Not present in females. Within normal variation. Not an adverse effect of treatment.
Adrenal weight (relative to body weight)	Increased (26%) in low dose male group.	Not present at the top dose. Within normal variation. No associated histopathology findings. Not an adverse effect of treatment.
Testes weights (absolute and relative to brain and body weight)	Increased 10%–15% in low dose male group.	Not present at the 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% decrease in mean body weights means a value of 70 g in test group animals versus 100 g in controls).

C.2 | Statistical analysis of the 90-day toxicity study on maize DAS1131 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 test diets (at low dose – 33% in Diet + control maize grain (064) at 17%, referred to as DAS1131 Low and high dose – 50% in Diet referred to as DAS1131 High) with those consuming the control diet. Continuous data were analysed separately for each variable and period or time interval, according to a Linear Mixed Model (factor: diet, sex and interaction 'dose-sex'; then, pairwise comparisons, between each test and control group (separately for each sex) were performed using a F-test (at the 5% level of significance). Ordinal multi-category data were analysed using a Cochran–Mantel–Haenszel (CMH) test. Binomial category data and unordered multi-category data were analysed by Fisher's exact probabilities test.

Missing data were considered by the Panel and found not to have an impact on the results (Table C.2).

TABLE C.2 Statistically significant findings in the 90-day toxicity study on maize DAS1131 in rats.

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
Body weight gain, food consumption, food conversion efficiency.	Increases and decreases during some phases of the study.	No consistent pattern. Overall, body weight gains within 5% of controls; food consumption within 2%. Within normal variation. Not an adverse effect of treatment.
FoB, movement duration in interval 5.	Low dose, increased in females (27%[35]); decreased in males (22%).	Not consistent, no significant change over whole measurement period. Not seen in the high dose animals. Not an adverse effect of treatment.
Absolute monocyte count	Reduced in the high dose groups, both sexes combined (25%) and in males (42%).	One top dose male had a value of zero. Other values within normal variation. No notable changes in other leukocyte counts. Not an adverse effect of treatment.
TSH	Decreased in low dose animals (15%), sexes combined.	All individual values are inside the concurrent control range. Not seen at the top dose. Not an adverse effect of treatment.
Kidney weight (relative to brain weight)	Decreased in low dose animals (6%) sexes combined.	Small magnitude. Not seen at the top dose. Not an adverse effect of treatment.
Prostate weight (Absolute and relative to brain and body weight)	Increased at the top dose (17%).	Low magnitude. No associated histopathology findings, no other changes in reproductive tract. Within normal variation. Not an adverse effect of treatment.
Thyroid weight	Increased at the top dose (13%) (both sexes combined).	Small magnitude. 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% decrease in mean body weights means a value of 70 g in test group animals vs. 100 g in controls).

[35]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 Cry1Da2 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	1.3	NA	NA
Layer	1.9	0.13	70	10	1.2	0.29	1.5
Turkey	7	0.50	50	NA	0.93	NA	NA
Breeding pigs	260	6	70	20	0.42	0.19	0.61
Finishing pigs	100	3	70	NA	0.55	NA	NA
Beef cattle[a]	500	12	80	80	0.50	0.81	1.3
Dairy cattle	650	25	30	60	0.30	0.97	1.3
Ram/ewe	75	2.5	30	NA	0.26	NA	NA
Lamb	40	1.7	30	30	0.33	0.54	0.87

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 DGT-28 EPSPS 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	2.3	NA	NA
Layer	1.9	0.13	70	10	2.3	0.41	2.7
Turkey	7	0.50	50	NA	1.7	NA	NA
Breeding pigs	260	6	70	20	0.76	0.28	1.0
Finishing pigs	100	3	70	NA	0.99	NA	NA
Beef cattle[a]	500	12	80	80	0.90	1.2	2.1
Dairy cattle	650	25	30	60	0.54	1.4	1.9
Ram/ewe	75	2.5	30	NA	0.47	NA	NA
Lamb	40	1.7	30	30	0.60	0.77	1.4

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.