Assessment of genetically modified maize DP4114 x MON 810 x MIR604 x NK603 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2018-150)

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Assessment of genetically modified maize DP4114 × MON 810 × MIR604 × NK603 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2018-150)

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Abstract

Maize DP4114 × MON 810 × MIR604 × NK603 (four-event stack maize) was produced by conventional crossing to combine four single events: DP4114, MON 810, MIR604 and NK603. The GMO Panel previously assessed the four single maize events and one of the subcombinations and did not identify safety concerns. No new data on the single maize events or the assessed subcombination were identified that could lead to modification of the original conclusions on their safety. The molecular characterisation, comparative analysis (agronomic, phenotypic and compositional characteristics) and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single maize events and of the newly expressed proteins in the four-event stack maize does not give rise to food and feed safety and nutritional concerns. The GMO Panel concludes that the four-event stack maize, is as safe as the comparator and the selected non-GM reference varieties. In the case of accidental release of viable grains of the four-event stack maize into the environment, this would not raise environmental safety concerns. The GMO Panel assessed the likelihood of interactions among the single events in nine of the maize subcombinations not previously assessed and concludes that these are expected to be as safe as the single events, the previously assessed subcombination and the four-event stack maize. Post-market monitoring of food/feed is not considered necessary. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of the four-event stack maize. The GMO Panel concludes that the four-event stack maize and its subcombinations are as safe as the non-GM comparator and the selected non-GM reference varieties with respect to potential effects on human and animal health and the environment.

Keywords: GMO, herbicide tolerant, maize (*Zea mays*), DP4114, MON 810, MIR604, NK603, import and processing

Requestor: Competent Authority of The Netherlands

Question number: EFSA-Q-2018-00370

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Summary

Following the submission of application EFSA-GMO-NL-2018-150 under Regulation (EC) No 1829/2003 from Pioneer Hi-Bred International, Inc. as represented by Pioneer Overseas Corporation referred to hereafter as 'the applicant', the Panel on Genetically Modified Organisms of the European Food Safety Authority (EFSA) (referred to hereafter as 'GMO Panel') was asked to deliver a Scientific Opinion on the safety of genetically modified (GM) glufosinate and glyphosate herbicides-tolerant and insect-resistant maize (*Zea mays* L.) DP4114 × MON 810 × MIR604 × NK603 (referred to hereafter as 'four-event stack maize') and its subcombinations independently of their origin, according to Regulation (EU) No 503/2013 (referred to hereafter as 'subcombinations'). The scope of application EFSA-GMO-NL-2018-150 is for import, processing and food and feed uses within the European Union (EU) of maize DP4114 × MON 810 × MIR604 × NK603 and all its subcombinations independently of their origin, and does not include cultivation in the EU. The term 'subcombination' refers to any combination of up to three of the events present in the four-event stack maize. The safety of subcombinations occurring as segregating progeny in the harvested grains of maize DP4114 × MON 810 × MIR604 × NK603 is evaluated in the context of the assessment of the four-event stack maize. The safety of subcombinations that have either been or could be produced by conventional crossing through targeted breeding approaches, and which can be bred, produced and marketed independently of the four-event stack, are risk assessed separately in the present scientific opinion. The four-event stack maize was produced by conventional crossing to combine four single maize events: DP4114 expressing Cry1F to confer resistance to lepidopteran pests, Cry34Ab1 and Cry35Ab1 to confer resistance to coleopteran pests, and PAT providing resistance to glufosinate-ammonium-containing herbicides; MON 810 expressing Cry1Ab to confer resistance to lepidopteran pests; MIR604 expressing mCry3A to confer resistance to coleopteran pests and PMI as a selectable marker; and NK603 expressing CP4 EPSPS and CP4 EPSPS L214P to confer tolerance to glyphosate-containing herbicides.

The GMO Panel evaluated the four-event stack maize and its subcombinations with reference to the scope and appropriate principles described in its applicable guidelines for the risk assessment of GM plants and the post-market environmental monitoring. The GMO Panel considered the information submitted in application EFSA-GMO-NL-2018-150, additional information provided by the applicant during the risk assessment, the scientific comments submitted by the Member States and the relevant scientific literature. For application EFSA-GMO-NL-2018-150, previous assessments of the four single events (DP4114, MON 810, MIR604 and NK603) and one of the subcombinations provided a basis for the assessment of the four-event stack maize and all its subcombinations. No safety concerns were identified by the GMO Panel in the previous assessments. No safety issue concerning the four single maize events was identified by the updated bioinformatic analyses, nor reported by the applicant since the publication of the previous GMO Panel scientific opinions. Therefore, the GMO Panel considers that its previous conclusions on the safety of the single maize events remain valid. For the four-event stack maize, the risk assessment included the molecular characterisation of the inserted DNA and analysis of protein expression. An evaluation of the comparative analysis of agronomic, phenotypic and compositional characteristics was carried out, and the safety of the newly expressed proteins and the whole food and feed were evaluated with respect to potential toxicity, allergenicity and nutritional characteristics. Environmental impacts and post-market environmental monitoring (PMEM) plan were also evaluated. The molecular characterisation data establish that the events DP4114, MON 810, MIR604 and NK603 combined in in the four-event stack maize have retained their integrity. Protein expression analysis showed that the levels of the newly expressed proteins are similar in the four-event stack maize and in the single events. The comparative analysis of agronomic and phenotypic characteristics and grain and forage composition identified no differences between maize DP4114 × MON 810 × MIR604 × NK603 and the non-GM comparator (referred to hereafter as comparator) that required further assessment except for the changes in early and final population, in carbohydrates, crude protein and phosphorus in forage. These changes were further assessed for food/feed safety and environmental impact and raised no concern. The molecular characterisation, the comparative analysis and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single maize events and of the newly expressed proteins in the four-event stack maize does not give rise to food and feed safety and nutritional concerns. The GMO Panel concludes that maize DP4114 × MON 810 × MIR604 × NK603, is as safe as the comparator and the selected commercial non-GM maize reference varieties (referred to hereafter as non-GM reference varieties). Considering the combined events and their potential interactions, the outcome of the comparative analysis, and the routes and levels of exposure, the GMO Panel concludes that maize

DP4114 × MON 810 × MIR604 × NK603 would not raise safety concerns in the case of accidental release of viable GM maize grains into the environment. Since no new safety concerns were identified for the previously assessed subcombination, and no new data leading to the modification of the original conclusions on safety were identified, the GMO Panel considers that its previous conclusions on this maize subcombination remain valid. For the remaining subcombinations included in the scope of application EFSA-GMO-NL-2018-150, no experimental data were provided. The GMO Panel assessed the possibility of interactions between the events in these subcombinations and concludes that these subcombinations would not raise safety concerns. These subcombinations are therefore expected to be as safe as the single events, the previously assessed subcombination and the four-event stack maize. Given the absence of safety concerns for foods and feeds from maize DP4114 × MON 810 × MIR604 × NK603 and its subcombinations, the GMO Panel considers that post-market monitoring of these products is not necessary. The PMEM plan and reporting intervals are in line with the intended uses of the four-event stack maize and its subcombinations. Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issue pertaining to the intended uses of maize DP4114 × MON 810 × MIR604 × NK603 and its subcombinations.

The GMO Panel concludes that maize DP4114 × MON 810 × MIR604 × NK603 and its subcombinations, as described in this application, are as safe as the comparator and the selected non-GM reference varieties with respect to potential effects on human and animal health and the environment.

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1. Introduction

The scope of the application EFSA-GMO-NL-2018-150 is for food and feed uses, import and processing of the genetically modified (GM) herbicide-tolerant and insect-resistant maize DP4114 × MON 810 × MIR604 × NK603 and all its subcombinations independently of their origin and does not include cultivation in the European Union (EU).

1.1. Background

On 8 May 2018, the European Food Safety Authority (EFSA) received from the Competent Authority of The Netherlands application EFSA-GMO-NL-2018-150 for authorisation of maize DP4114 × MON 810 × MIR604 × NK603 (hereafter referred to as 'the four-event stack maize') (Unique Identifier DP-ØØ4114-3, MON-ØØ81Ø-6, SYN-IR6Ø4-5, MON-ØØ6Ø3-6), submitted by Pioneer Hi-Bred International, Inc. as represented by Pioneer Overseas Corporation (hereafter referred to as 'the applicant') according to Regulation (EC) No 1829/2003[1]. Following receipt of application EFSA-GMO-NL-2018-150, EFSA informed EU Member States and the European Commission, and made the application available to them. Simultaneously, EFSA published the summary of the application.[2]

EFSA checked the application for compliance with the relevant requirements of Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013[3] and, when needed, asked the applicant to supplement the initial application. On 10 August 2018, EFSA declared the application valid.

From the validity date, EFSA and its scientific Panel on Genetically Modified Organisms (hereafter referred to as 'the GMO Panel') endeavoured to respect a time limit of 6 months to issue a scientific opinion on application EFSA-GMO-NL-2018-150. Such time limit was extended whenever EFSA and/or its GMO Panel Requested supplementary information to the applicant. According to Regulation (EC) No 1829/2003, any supplementary information provided by the applicant during the risk assessment was made available to the EU Member States and European Commission (for further details, see the Section 'Documentation', below). In accordance with Regulation (EC) No 1829/2003, EFSA consulted the nominated risk assessment bodies of EU Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC.[4] The EU Member States had 3 months to make their opinion known on application EFSA-GMO-NL-2018-150 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 DP4114 × MON 810 × MIR604 × NK603 in the context of its scope as defined in application EFSA-GMO-NL-2018-150.

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 GMO Panel based its scientific assessment of the four-event stack maize on the valid application EFSA-GMO-NL-2018-150, 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 additional unpublished studies submitted by the applicant in order to comply with the specific

[1] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1–23.
[2] https://open.efsa.europa.eu/questions/EFSA-Q-2018-00370
[3] Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. OJ L 157, 8.6.2013, p. 1–48.
[4] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 12.3.2001, p. 1–38.
[5] These particulars are available online at: https://open.efsa.europa.eu/questions/EFSA-Q-2018-00370

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,b, 2015; EFSA Scientific Committee, 2011) and explanatory notes and statements (i.e. EFSA GMO Panel, 2010b; EFSA, 2010, 2014, 2017a,b, 2019a,b) for the risk assessment of GM plants.

For this application, in the context of the contracts [OC/EFSA/GMO/2018/04, OC/EFSA/GMO/2018/02 and EOI/EFSA/SCIENCE/2020/01 – CT02GMO] the contractors performed preparatory work for the evaluation of the applicant`s literature search, methods applied for the statistical analysis and statistical analysis of the 90-day toxicity study on maize DP4114 × MON 810 × MIR604 × NK603.

3. Assessment

3.1. Introduction

Application EFSA-GMO-NL-2018-150 covers the four-event stack maize DP4114 × MON 810 × MIR604 × NK603 and all its 10 subcombinations independently of their origin (Table 1).

Table 1: Eleven combinations of the events covered by the scope of application EFSA-GMO-NL-2018-150

Degree of stacking	Events	Unique identifiers
Four-event stack	MON 810 × MIR604 × NK603 × DP4114	MON-ØØ81Ø-6, SYN-IR6Ø4-5, MON-ØØ6Ø3-6, DP-ØØ4114-3
Three-event stack	MIR604 × NK603 × DP4114	SYN-IR6Ø4-5, MON-ØØ6Ø3-6, DP-ØØ4114-3
	MON 810 × NK603 × DP4114	MON-ØØ81Ø-6, MON-ØØ6Ø3-6, DP-ØØ4114-3
	MON 810 × MIR604 × DP4114	MON-ØØ81Ø-6, SYN-IR6Ø4-5, DP-ØØ4114-3
	MON 810 × MIR604 × NK603	MON-ØØ81Ø-6, SYN-IR6Ø4-5, MON-ØØ6Ø3-6
Two-event stack	NK603 × DP4114	MON-ØØ6Ø3-6, DP-ØØ4114-3
	MIR604 × DP4114	SYN-IR6Ø4-5, DP-ØØ4114-3
	MIR604 × NK603	SYN-IR6Ø4-5, MON-ØØ6Ø3-6
	MON 810 × DP4114	MON-ØØ81Ø-6, DP-ØØ4114-3
	MON 810 × NK603	MON-ØØ81Ø-6, MON-ØØ6Ø3-6
	MON 810 × MIR604	MON-ØØ81Ø-6, SYN-IR6Ø4-5

The term 'subcombination' refers to any combination of up to three of the maize events DP4114, MON 810, MIR604 and NK603.

The safety of subcombinations occurring as segregating progeny in harvested grains of maize DP4114 × MON 810 × MIR604 × NK603 is evaluated in the context of the assessment of the four-event stack maize in Section 3.5 of the present scientific opinion.

'Subcombination' also covers combinations that have either been or could be produced by conventional crossing through targeted breeding approaches (EFSA GMO Panel, 2011a). These are maize stacks that can be bred, produced and marketed independently of the four-event stack maize. These subcombinations are assessed in Section 3.5 of this scientific opinion.

The four-event stack maize was produced by conventional crossing to combine four single maize events: DP4114 expressing Cry1F to confer resistance to lepidopteran pests, Cry34Ab1 and Cry35Ab1 to confer resistance to coleopteran pests, and PAT providing resistance to glufosinate-ammonium-containing herbicides; MON 810 expressing Cry1Ab to confer resistance to lepidopteran pests; MIR604 expressing mCry3A to confer resistance to coleopteran pests and PMI as selectable marker; and NK603 expressing CP4 EPSPS and CP4 EPSPS L214P to confer tolerance to glyphosate-containing herbicides.

All four single events were assessed previously (see Table 2) and no safety concerns were identified.

Table 2: Single maize events and subcombination of maize DP4114 × MON 810 × MIR604 × NK603 previously assessed by the GMO Panel

Events	Application or mandate	Reference
MON 810	RX-MON 810	EFSA (2009a)
MIR604	AP 11	EFSA (2009b)
	RX-013	EFSA GMO Panel (2019a)
NK603	Art4_NK603	EFSA (2004)
	CE/ES/00/01	EFSA (2007)
	AP 22	EFSA (2009c)
	RX-NK603	EFSA (2009d)
DP4114	AP 123	EFSA GMO Panel (2018a)
MON 810 × NK603	AP 01	EFSA (2005a)
	C/GB/02/M3/3	EFSA (2005b)
	AP 92	EFSA GMO Panel (2017a)
	AP 127	EFSA GMO Panel (2021a)
	RX-007	EFSA GMO Panel (2018b)

3.2. Updated information on single events

Since publication of the scientific opinions on the single maize events by the GMO Panel (see Table 2), no safety issue concerning the four single events has been reported by the applicant.

Updated bioinformatic analyses for events DP4114, MON 810, MIR604 and NK603 confirmed that no known endogenous genes were disrupted by event DP4114, MIR604 and NK603. This bioinformatic analysis also confirmed that in the case of MON 810 a possible deletion or rearrangement happened at the insertion site. However, the assessment performed in the frame of application EFSA-GMO-NL-2015-127 and EFSA-GMO-RX-17 showed that the genes present in this region are normally expressed in MON 810 (EFSA GMO Panel, 2021a,b).

Updated bioinformatic analyses of the amino acid sequence of the newly expressed Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI, CP4 EPSPS and CP4 EPSPS L214P proteins confirmed previous results indicating no significant similarities to known toxins and allergens. Updated bioinformatic analyses of the newly created open reading frames (ORFs) within the inserts or spanning the junctions between the insert and the flanking regions for events DP4114, MON 810, MIR604 and NK603 confirmed previous analyses (Table 2). These analyses indicate that the production of a new peptide showing significant similarities to toxins or allergens for any of the events in maize DP4114 × MON 810 × MIR604 × NK603 is highly unlikely.

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination, the applicant performed a sequence identity analysis with microbial DNA for maize events DP4114, MON 810, MIR604 and NK603. The likelihood and potential consequences of plant-to-bacteria gene transfer are described in Section 3.4.4.2.

Based on the above information, the GMO Panel considers that its previous conclusions on the safety of the single maize events remain valid.

3.3. Systematic literature review

The GMO Panel assessed the applicant's literature searches on maize DP4114 × MON 810 × MIR604 × NK603, which include a scoping review, according to the guidelines given in EFSA (2010, 2017a). A systematic review as referred to in Regulation (EU) No 503/2013 has not been provided in support to the risk assessment of application EFSA-GMO-NL-2018-150. Based on the outcome of the scoping review, the GMO Panel agrees that there is limited value of undertaking a systematic review for maize DP4114 × MON 810 × MIR604 × NK603 at present.

The overall quality of the performed literature searches is acceptable.

None of the relevant publications identified through the literature searches reported information pointing to safety issues associated with the intended uses of maize DP4114 × MON 810 × MIR604 × NK603 and its subcombinations.

3.4. Risk assessment of the four-event stack maize DP4114 × MON 810 × MIR604 × NK603

3.4.1. Molecular characterisation[6]

In line with the requirements laid down by Regulation (EU) 503/2013, the possible impact of the combination of the events on the integrity of the events, the expression levels of the newly expressed proteins or the biological functions conferred by the individual inserts are considered below.

3.4.1.1. Genetic elements and biological function of the inserts

Maize events DP4114, MON 810, MIR604 and NK603 were combined by conventional crossing to produce the four-event stack maize DP4114 × MON 810 × MIR604 × NK603. The structure of the inserts introduced into the four-event stack maize are described in detail in the respective EFSA scientific opinions (Table 2) and no new genetic modifications were involved. Genetic elements in the expression cassettes of the single events are summarised in Table 3.

Intended effects of the inserts in maize DP4114 × MON 810 × MIR604 × NK603 are summarised in Table 4. Based on the known biological function of the newly expressed proteins (Table 4), the only foreseen interactions at the biological level are between the five Cry proteins in susceptible insects.

Table 3: Genetic elements in the expression cassettes of the events stacked in maize DP4114 × MON 810 × MIR604 × NK603

Event	Promoter	5′ UTR	Transit peptide	Coding region	Terminator
DP4114	*ubi*ZM1 (*Zea mays*)	*ubi*ZM1-5′UTR/*ubi*ZM1-Intron (*Zea mays*)	–	*cry1F** (*Bacillus thuringiensis*)	ORF25 (*Agrobacterium tumefaciens*)
	*ubi*ZM1 (*Zea mays*)	*ubi*ZM1-5′UTR/*ubi*ZM1-Intron (*Zea mays*)	–	*cry34Ab1** (*Bacillus thuringiensis*)	*pin*II (*Solanum tuberosum*)
	TA Peroxidase (*Triticum aestivum*)	–	–	*cry35Ab1** (*Bacillus thuringiensis*)	*pin*II (*Solanum tuberosum*)
	35S (CaMV)	–	–	*pat** (*Streptomyces viridochromogenes*)	35S (CaMV)
MON 810	35S (CaMV)	I-*Hsp70* (*Zea mays*)	–	*cry1Ab* (*Bacillus thuringiensis*) (partial)	(deleted during the integration)
MIR604	MTL (*Zea mays*)	–	–	*mcry*3A* (*Bacillus thuringiensis*)	nos (*Agrobacterium tumefaciens*)
	ZmUbiInt (*Zea mays*)	–	–	*pmi* (*Escherichia coli*)	nos (*Agrobacterium tumefaciens*)
NK603	*act1* (*Oryza sativa*)	*act1* intron (*Oryza sativa*)	CTP2 (*Arabidopsis thaliana*)	CP4 *epsps* (*Agrobacterium tumefaciens*)	nos (*Agrobacterium tumefaciens*)
	35S (CaMV)	I-*Hsp70* (*Zea mays*)	CTP2 (*Arabidopsis thaliana*)	CP4 *epsps l214p* (*Agrobacterium tumefaciens*)	nos (*Agrobacterium tumefaciens*)

–: When no element was specifically introduced to optimise expression.
*: Codon optimised.

[6] Dossier: Part II – Section 1.2 and additional information 21/1/2019, 23/09/2019 and 5/8/2020.

Table 4: Characteristics and intended effects of the events stacked in maize DP4114 × MON 810 × MIR604 × NK603

Event	Protein	Donor organism and biological function	Intended effects in GM plant
DP4114	Cry1F	Based on genes from *Bacillus thuringiensis* subsp. *aizawai*. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal protein (cry) genes (Schnepf et al., 1998; Ellis et al., 2002).	Maize DP4114 expresses a truncated version of the Cry1F protein. Cry1F is a protein toxic to certain lepidopteran larvae feeding on maize.
	Cry34Ab1	Based on genes from *Bacillus thuringiensis* strain PS149B1. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal protein (cry) genes (Schnepf et al., 1998; Ellis et al., 2002).	Maize DP4114 expresses the Cry34Ab1. In complex with Cry35Ab1 this protein is toxic to certain coleopteran larvae feeding on maize.
	Cry35Ab1	Based on genes from *Bacillus thuringiensis* strain PS149B1. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal protein (cry) genes (Schnepf et al., 1998; Ellis et al., 2002).	Maize DP4114 expresses the Cry35Ab1. In complex with Cry34Ab1 this protein is toxic to certain coleopteran larvae feeding on maize.
	PAT	Based on a gene from *Streptomyces viridochromogenes* Tü494. Phosphinothricin-acetyl-transferase (PAT) enzyme acetylates L-glufosinate-ammonium (Thompson et al., 1987; Wohlleben et al., 1988; Eckes et al., 1989).	Maize DP4114 expresses the PAT protein which confers tolerance to glufosinate-ammonium-containing herbicides (Droge-Laser et al., 1994).
MON 810	Cry1Ab	Based on a gene *from Bacillus thuringiensis* subsp. *kumamotoensis*. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal protein (cry) genes (Schnepf et al., 1998).	Event MON 810 expresses a chimeric, truncated cry1Ab gene. Cry1Ab is a chimeric protein toxic to certain lepidopteran larvae feeding on maize.
MIR604	mCry3A	Based on genes from *Bacillus thuringiensis* subsp. *tenebrionis*. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal proteins (Cry) (Schnepf et al., 1998; Ellis et al., 2002).	Event MIR604 expresses a modified version of the native Cry3A protein (Chen and Stacey, 2003). mCry3A is a protein toxic to certain coleopteran larvae feeding on maize.
	PMI	Based on a gene from *E. coli*. PMI (phosphomannose isomerase) catalyses the isomerisation of mannose-6-phosphate to fructose-6-phosphate and plays a role in the metabolism of mannose (Markovitz et al., 1967).	Event MIR604 expresses PMI, which is used as selectable marker. Mannose normally inhibits root growth, respiration and germination. Transformed cells expressing PMI are able to utilise mannose as a carbon source (Negrotto et al., 2000).
NK603	CP4 EPSPS	Based on a gene from *Agrobacterium* strain CP4 (Barry et al., 2001). 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) is an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Herrmann, 1995).	Event NK603 expresses the bacterial CP4 EPSPS protein which confers tolerance to glyphosate-containing herbicides as it has lower affinity towards glyphosate than the plant endogenous enzyme.
	CP4 EPSPS L214P	Based on a gene from *Agrobacterium* strain CP4 (Barry et al., 2001). 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) is an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Herrmann, 1995).	Event NK603 expresses also CP4 EPSPS L214P – this variant, compared to the CP4 EPSPS protein, contains a single amino acid substitution from leucine to proline at position 214. The two CP4 EPSPS protein variants are structurally and functionally equivalent.

3.4.1.2. Integrity of the events in the four-event stack

The genetic stability of the inserted DNA over multiple generations in the single maize events DP4114, MON 810, MIR604 and NK603 was demonstrated previously (Table 2 and Section 3.2). Integrity of these events in maize DP4114 × MON 810 × MIR604 × NK603 was demonstrated by Southern analyses and by polymerase chain reaction (PCR) followed by sequence analysis demonstrating that the sequences of the events (inserts and their flanking regions) in the four-event maize stack are identical to the sequences already assessed (Table 2 and Section 3.2), thus confirming that the integrity of these events was maintained in the four-event stack maize.

3.4.1.3. Information on the expression of the inserts

Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI and CP4 EPSPS protein levels were analysed by enzyme-linked immunosorbent assay (ELISA) in material harvested from a field trial at four locations in the USA in 2015. Samples analysed included leaves (16, 19 and 63–65 BBCH growth stages), roots (16, 19 and 63–65 BBCH growth stages), pollen (63–65 BBCH growth stage), stalk (63–65 BBCH growth stage), whole plant (63–65 BBCH growth stage), forage (85 BBCH growth stage) and grain (87–99 BBCH growth stage), both those treated and not treated with intended herbicides.

In order to assess the changes in protein expression levels which may result from potential interactions between the events, protein levels were determined for the four-event stack and the corresponding single events in different parts of the plant.

The levels of all the newly expressed proteins in the four-event stack and the corresponding singles were comparable in all tissues (Appendix B). Therefore, there is no indication of an interaction that may affect the levels of the newly expressed proteins in this stack.

3.4.1.4. Conclusion of the molecular characterisation

The molecular data establish that the events stacked in maize DP4114 × MON 810 × MIR604 × NK603 have retained their integrity. Protein expression analyses showed that the levels of the newly expressed proteins are similar in the four-event stack and in the single events. Therefore, there is no indication of an interaction that may affect the integrity of the events and the levels of the newly expressed proteins in this stack.

Based on the known biological function (Table 3) of the newly expressed proteins, the only potential functional interactions are among the Cry proteins in susceptible insects, which will be dealt with in Section 3.4.4.

3.4.2. Comparative analysis[7]

3.4.2.1. Overview of studies conducted for the comparative analysis

Application EFSA-GMO-NL-2018-150 presents data on agronomic and phenotypic characteristics, as well as on forage and grain composition of maize DP4114 × MON 810 × MIR604 × NK603 (Table 5).

Table 5: Overview of the comparative analysis studies to characterise the four-event stack maize in application EFSA-GMO-NL-2018-150

Study focus	Study details	Comparator	Non-GM reference varieties
Agronomic and phenotypic analysis	Field study, USA, 2015, ten sites(a)	P0751	Twenty(c)
Compositional analysis	Field study, USA, 2015, eight sites(b)		

(a): Field trials were located in Indiana, Kansas, Minnesota, Nebraska, Oklahoma and Texas, and two field trials in Iowa and Illinois.
(b): Field trials were located in in Indiana, Kansas, Minnesota and Nebraska, and two field trials in Iowa and Illinois.
(c): Non-GM maize varieties used in the agronomic, phenotypic and compositional field trials, with their corresponding relative maturity indicated in brackets were 35F38 (105), 36B08 (105), 35P12 (105), 35K02 (106), 34Y02 (108), P0965 (109), 34B39 (109), 34F06 (110), 34H31 (110), 33W82 (111), P1184 (111), P1319 (113), 3335 (113), P1395 (113), XL5246 (105), XL5354 (107), XL5475 (108), XL5435 (109), XL6077 (111), XL6272 (112). 36B08 was used for the agronomic and phenotypic analysis only.

[7] Dossier: Part II – Section 1.3 and additional information 6/9/2018, 5/11/2018, 8/2/2019, 11/6/2019; spontaneous information 17/12/2019.

3.4.2.2. Experimental field trial design and statistical analysis

The materials grown at each field trial site were: the four-event stack maize exposed to the intended glyphosate- and glufosinate-ammonium-containing herbicides (treated), the four-event stack maize not exposed to the intended herbicides (untreated), the comparator P0751 and four of the twenty commercial non-GM hybrid maize reference varieties (hereafter 'non-GM reference varieties').

The agronomic/phenotypic and compositional data were analysed as specified by EFSA GMO Panel (2010b, 2011a). This includes, for each of the two treatments of the four-event stack maize, the application of a difference test (between the GM maize and the comparator) and an equivalence test (between the GM maize and the set of non-GM reference varieties).[8] The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).[9]

3.4.2.3. Suitability of selected test materials

Selection of the GM maize line and comparator

To produce the four-event stack maize, the single events DP4114, MON 810, MIR604 and NK603 were transferred in the genetic background of two different non-GM inbred lines, PHW2Z and PHR1J.

In subsequent subsections, GM maize DP4114 × MON 810 × MIR604 × NK603 refers to hybrid (F_1 generation) obtained crossing GM inbred line PHR1J (carrying NK603) with GM inbred line PHW2Z (carrying DP4114 × MON 810 × MIR604).

The comparator selected in the field trials is the hybrid maize P0751 that was obtained by crossing the non-GM inbred lines PHR1J and PHW2Z`. As documented by the pedigree, the GMO Panel considers the produced comparator suitable for the comparative analysis.

Both maize DP4114 × MON 810 × MIR604 × NK603 and its comparator belong to a comparative relative maturity (CRM) of 107, which is considered appropriate for growing in environments across North America, where the comparative field trials were conducted.

Selection of non-GM reference varieties

The 20 non-GM reference varieties with a relative maturity ranging from 105 to 113 were selected by the applicant and at each field trial site four of them were tested (see Table 5). On the basis of the information provided on relative maturity classes and year of commercialisation, the GMO Panel considers the selected non-GM reference varieties appropriate for the comparative assessment.

Seed production and quality

Seeds of four-event stack maize and the comparator were produced, harvested and stored under similar conditions, before being sown in the field trials. The seed lots of the four-event stack maize and the comparator were verified for their identity via event specific polymerase chain reaction analysis. The germination of four-event stack maize and the comparator was tested following the AOSA[10] test protocol. The GMO Panel considers that the starting seed used as test material in the agronomic, phenotypic and compositional studies was of adequate quality.

Conclusion on suitability

The GMO Panel is of the opinion that the four-event stack maize, its comparator and the non-GM reference varieties were properly selected and of adequate quality. Therefore, the test materials are considered suitable for the comparative analysis.

3.4.2.4. Representativeness of the receiving environments

Selection of field trial sites

The selected field trial sites were located in commercial maize-growing regions of the US. Climate and soil characteristics of the selected fields were diverse,[11] corresponding to optimal, near optimal

[8] The purpose of the test of equivalence is to evaluate the estimated mean values for the GM crop taking into account natural variability as defined by a set of non-GM references varieties with a history of safe use for consumption as food or feed.

[9] 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).

[10] AOSA. 2012. Rules for testing seeds. Association of Official Seed Analysts,

[11] Soil types of the field trials were clay loam, silty clay loam, loam, silt loam and sandy loam; soil organic carbon ranged from 0.5% to 2.4%; soil pH ranged from 5.5 to 7.3. Average temperatures and sum of precipitations during the usual crop growing season ranged, respectively, from 17.4°C to 23.2°C and from 319 to 762 mm.

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.

Meteorological conditions

Maximum and minimum mean temperatures and sum of precipitations were provided on a daily basis. Exceptional weather conditions were reported at four of the selected sites.[12] 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 analyses.

Management practices

The field trials included plots containing the four-event stack maize, plots with the comparator and plots with non-GM reference varieties, all managed according to local agricultural practices. In addition, the field trials included plots containing the four-event stack maize managed following the same agricultural practices, but where the conventional herbicide regime was replaced applying the intended herbicides. In particular, the conventional herbicide regime was replaced with two applications of the glyphosate- and glufosinate-ammonium-containing herbicides that were applied at BBCH 14–17 growth stage. Despite not considered a common agricultural practice, low seeding rates were applied at all field trials, resulting in low early and final plant populations. However, the sowing rates were within the recorded rates of the primary US maize production states (USDA, 2010). The GMO Panel considers that the management practices including sowing, harvesting and application of plant protection products were acceptable for the field trials.

Conclusion on representativeness

The GMO Panel concludes that the geographical locations, soil and climate characteristics, meteorological conditions and most of the management practices of the field trials are typical of the receiving environments where the test materials could be grown.

3.4.2.5. Agronomic and phenotypic analysis

Ten agronomic and phenotypic endpoints[13] plus information on abiotic stressors, disease incidence and arthropod damage were collected from the field trials (Table 5). The endpoints ear count and lodging were not analysed as described in Section 3.4.2.2 because of insufficient variability in the data.

The outcome of the analysis for the remaining eight endpoints was as follows:

- For the four-event stack maize (not treated with the intended herbicides), statistically significant differences with the comparator were identified for early population, plant height, final population, grain moisture and yield. Early and final population fell under equivalence category IV, while the other endpoints fell under equivalence category I.[14]
- For the four-event stack maize (treated with the intended herbicides), statistically significant differences with the comparator were identified for early population, plant height, days to maturity, final population, grain moisture and yield. Early and final population fell under equivalence category III, while the other endpoints fell under equivalence category I.[14]

Early and final population for the four-event stack maize were reduced with respect to the comparator and the non-GM reference varieties (equivalence category III-IV). As the values for the other yield components were within the range of natural variability (yield and test weight fell under equivalence category I), the GMO Panel considered that these differences do not affect the use of the field trial data for the comparative analysis. Whether the differences can lead to an environmental adverse effect is considered in Section 3.4.4.1.

[12] Excessive rain from tropical storm reported at site in Indiana, severe wind and rain storm at the site in Kansas, severe wind storm at the site in Nebraska and wind damage reported in one plot at the site in Oklahoma.

[13] Early population, days to flowering, plant height, days to maturity, lodging, ear count, final population, grain moisture, 100-kernel weight, yield.

[14] For early stand count (plants/m^2), the estimated mean values were 4.57 (untreated GM), 4.63 (treated GM), 4.9 (comparator) and 4.95 (reference varieties); the equivalence interval was 4.67–5.24. For final stand count (plants/m^2), the estimated mean values were 4.49 (untreated GM), 4.58 (treated GM), 4.79 (comparator) and 4.88 (reference varieties); the equivalence interval was 4.6–5.15.

3.4.2.6. Compositional analysis

Maize forage and grains harvested from the field trial study in the USA in 2015 (Table 5) were analysed for 81 constituents (10 in forage and 71 in grain), including the key constituents recommended by OECD (2002). The statistical analysis was not applied to 11 grain constituents[15] because their concentration in more than half of the observations were below the limit of quantification.

The statistical analysis was applied to the remaining 70 constituents (10 in forage[16] and 60 in grain[17]); a summary of the outcome of the test of difference and the test of equivalence is presented in Table 6.

- For the four-event stack maize (not treated with the intended herbicides), significant differences with the comparator were identified for 29 endpoints (1 in forage and 28 in grain); of those, phosphorus in forage fell under equivalence category IV (Table 7), while the other endpoints fell under category I/II.
- For the four-event stack maize (treated with the intended herbicides), significant differences with the comparator were identified for 51 endpoints (5 in forage and 46 in grain); three forage endpoints fell under equivalence category III/IV (Table 7), while the other endpoints fell under category I/II.

Table 6: Outcome of the comparative compositional analysis of grains and forage from maize DP4114 × MON 810 × MIR604 × NK603. The table shows the number of endpoints in each category

	Test of difference[a]			
	Not treated[c]		**Treated[c]**	
	Not different	**Significantly different**	**Not different**	**Significantly different**
Test of equivalence[b]				
Category I/II	40	28[d]	18	48[d]
Category III/IV	_[e]	1[f]	_[e]	3[f]
Not categorised	1[g]	_[h]	1[g]	_[h]
Total endpoints	70		70	

(a): Comparison between the four-event stack maize and the comparator.
(b): 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.
(c): Treated with the intended glyphosate- and glufosinate-ammonium-containing herbicides.
(d): Endpoints with significant differences between the four-event stack maize and its comparator and falling in equivalence category I-II. For grains, for both treated and non-treated GM: arginine, aspartic acid, glycine, histidine, lysine, phenylalanine, proline, threonine, tyrosine, palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), eicosenoic acid (C20:1), copper, iron, magnesium, phosphorus, potassium, ferulic acid, p-coumaric acid, trypsin inhibitor, ash, NDF, beta carotene, γ-tocopherol, pyridoxine. Only non-treated: methionine, raffinose. Only treated: ADF, TDF, carbohydrates, crude fibre, crude protein, alanine, cystine, glutamic acid, isoleucine, leucine, serine, tryptophan, valine, palmitoleic acid (C16:1), α-linolenic acid (C18:3), manganese, zinc, phytic acid, total tocopherols, thiamine. For forage, treated only: moisture, NDF.
(e): Endpoints with no significant differences between the four-event stack maize and its comparator and falling in equivalence category III/IV: none.

[15] lauric acid (C12:0), myristic acid (C14:0), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), eicosadienoic acid (C20:2), behenic acid (C22:0), erucic acid (C22:1), riboflavin, β-tocopherol, δ-tocopherol and furfural.
[16] Ash, calcium, carbohydrates, crude fat, crude fibre, crude protein, moisture, phosphorus, acid detergent fibre (ADF), neutral detergent fibre (NDF).
[17] Proximates 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 (α-tocopherol, ß-carotene, 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), 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), lignoceric acid (C24:0)) and other compounds (ferulic acid, inositol, *p*-coumaric acid, phytic acid, raffinose, trypsin inhibitor).

(f): Endpoints with significant differences between the four-event stack maize and its comparator and falling in equivalence category III/IV: For grains, none. For forage, treated only: carbohydrates, crude protein. Both treated and untreated: phosphorus. Quantitative results are reported in Table 7.
(g): Endpoints not categorised for equivalence and without significant differences between the four-event stack maize and its comparator: sodium in grain (both treated and not treated).
(h): Endpoints not categorised for equivalence and with significant differences between the four-event stack maize and its comparator: none.

The GMO Panel assessed all the significant differences between the four-event stack maize and its comparator, taking into account the potential impact on plant metabolism and the natural variability observed for the set of non-GM maize reference varieties. Quantitative results for the endpoints showing significant differences between the four-event stack maize and its comparator and falling under equivalence category III/IV are given in Table 7.

Table 7: Quantitative results (estimated means and equivalence limits) for compositional endpoints in forage that are further assessed based on the results of the statistical analysis

Endpoint	Maize DP4114 × MON 810 × MIR604 × NK603		Comparator	Non-GM reference varieties	
	Not treated[(a)]	Treated[(a)]		Mean	Equivalence limits
Crude protein (% dw)	8.87	9.56*	8.74	8.41	7.41–9.42
Carbohydrates (% dw)	82.9	82.0*	83.0	83.5	82.2–84.8
Phosphorus (% dw)	0.364*	0.384*	0.328	0.302	0.266–0.339

dw: dry weight.
For the four-event stack maize, 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), light grey (equivalence category III) and dark grey (equivalence category IV).
(a): Treated with the intended glyphosate- and glufosinate-ammonium-containing herbicides.

3.4.2.7. Conclusions on the comparative analysis

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

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

- None of the differences identified in agronomic and phenotypic characteristics between the four-event stack maize and the comparator needs further assessment except for the changes in early and final population. These endpoints are discussed for potential environmental impact in Section 3.4.4.1.
- None of the differences identified in forage and grain composition between the four-event stack maize and the comparator needs further food/feed safety assessment except for the changes in forage levels of crude protein, carbohydrates and phosphorus. These differences are further discussed in Section 3.4.3.

3.4.3. Food/Feed safety assessment

3.4.3.1. Effects of processing

Maize DP4114 × MON 810 × MIR604 × NK603 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 four-event stack maize into food and feed products is not expected to result in products being different from those of conventional non-GM maize varieties.

3.4.3.2. Stability of newly expressed proteins

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, 2017b, 2021c). The term protein stability encompasses several properties such as thermal stability, pH-dependent stability,

proteolytic stability and physical stability (e.g. tendency to aggregate), among others (Li et al., 2019). It has been shown, for example, that when characteristics of known food allergens are examined, one prominent trait attributed to food allergens is protein stability (Helm, 2001; Breiteneder and Mills, 2005; Costa et al., 2021).

Effect of temperature and pH on newly expressed proteins

The effects of temperature and pH on the Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, CP4 EPSPS (including its variant CP4 EPSPS L214P), mCry3 and PMI proteins have been previously evaluated by the GMO Panel (Table 2). No new information has been provided in the context of this application.

In vitro protein degradation by proteolytic enzymes

The resistance to degradation by pepsin of the newly expressed Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, CP4 EPSPS (including its variant CP4 EPSPS L214P), mCry3 and PMI proteins have been previously evaluated by the GMO Panel (Table 2). No new information has been provided in the context of this application.

3.4.3.3. Toxicology

Testing of newly expressed proteins

Eight proteins (Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, CP4 EPSPS and its variant CP4 EPSPS L214P, mCry3 and PMI) are newly expressed in the four-event stack maize DP4114 × MON 810 × MIR604 × NK603 (Section 3.4.1). The GMO Panel has previously assessed these proteins in the context of the single maize events (Table 2), and no safety concerns were identified for humans and animals. The GMO Panel is not aware of any other new information that would change its previous conclusions on the safety of these proteins. The potential for a functional interaction among the proteins newly expressed in maize DP4114 × MON 810 × MIR604 × NK603 has been assessed with regard to human and animal health. The five insecticidal proteins Cry1F, Cry34Ab1, Cry35Ab1, Cry1Ab and mCry3 are delta-endotoxins acting through cellular receptors found in target insect species. It is reported that the gastrointestinal tract of mammals, including humans, lacks receptors with high specific affinity to Cry proteins (Hammond et al., 2013; Koch et al., 2015; Jurat-Fuentes and Crickmore, 2017). The three enzymatic proteins (CP4 EPSPS and its variant CP4 EPSPS L214P, PAT and PMI) catalyse distinct biochemical reactions, acting on unrelated substrates and are not expected to interact. The CP4 EPSPS and its variant CP4 EPSPS L214P act on the shikimic acid pathway for the biosynthesis of aromatic amino acids in plants, showing high substrate specificity. The PAT enzyme acts on the glufosinate-ammonium-containing herbicides and the PMI enzyme catalyses the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. On the basis of the known biological function of the individual newly expressed proteins (Table 4), there is currently no expectation for their possible interactions relevant to the food and feed safety of the four-event stack maize DP4114 × MON 810 × MIR604 × NK603.

The GMO Panel concludes that there are no safety concerns to human and animal health related to the newly expressed proteins Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, CP4 EPSPS and its variant CP4 EPSPS L214P, mCry3 and PMI in the four-event stack maize DP4114 × MON 810 × MIR604 × NK603.

Testing of new constituent other than proteins

No new constituents other than newly expressed proteins have been identified in seed and forage from four-event stack maize DP4114 × MON 810 × MIR604 × NK603. Therefore, no further food/feed safety assessment of components other than the newly expressed proteins is required.

Information on altered levels of food and feed constituent

No altered levels of food/feed constituents have been identified in seed and forage from maize DP4114 × MON 810 × MIR604 × NK603 except forage levels of crude protein, carbohydrates and phosphorus. 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 in provided in Section 3.4.3.6.

Testing of the whole genetically modified food and feed

Based on the outcome of the molecular characterisation, comparative analysis and toxicological assessment, no indication of findings relevant to food/feed safety related to the stability and expression of the inserts or to interaction between the transformation events, and no modifications of toxicological concern in the composition of maize DP4114 × MON 810 × MIR604 × NK603 have been identified (see Sections 3.4.1, 3.4.2 and 3.4.3.3). Therefore, animal studies on food/feed derived from this four-event stack maize are not necessary (EFSA GMO Panel, 2011a). In accordance to Regulation (EU) No 503/2013, the applicant provided a 90-day oral repeated-dose toxicity study in rats on whole food and feed from each of the maize single event composing the four-event stack maize. In addition, the applicant provided also a 90-day oral repeated-dose toxicity study with maize DP4114 × MON 810 × MIR604 × NK603, which considered by the GMO Panel (Appendix A).

90-day studies on maize DP4114, maize MON 810 and maize NK603

The GMO Panel had previously concluded that these studies are in line with Regulation (EU) No 503/2013 and do not show adverse effects related to diets incorporating the single-event maize DP4114 (EFSA GMO Panel, 2018a), MON 810 or NK603 (EFSA GMO Panel, 2021a).

90-day studies on maize MIR 604

A 90-day study on maize MIR604 has been previously assessed by the GMO Panel in the context of the single-event renewal application dossier (EFSA GMO Panel, 2019a), and was not consider adequate because it was not possible to confirm the adequacy of the control material (i.e. to exclude it was a negative segregant, EFSA GMO Panel, 2011a; EFSA Scientific Committee, 2011); upon EFSA's request to fulfil the requirements of Regulation (EU) No 503/2013, the applicant provided a new 90-day toxicity study on maize MIR604.

In this new study, pair-housed RccHan:WIST Han Wistar rats (16/sex per group; 2 rats/cage) were allocated to two groups using a randomised complete block design with eight replications/sex. Groups were fed diets containing 50% of milled grains from maize MIR604 (test material) or the comparator (NP2392/NP2222, control material).

The study was adapted from OECD test guideline 408 (OECD, 2018), aligned with EFSA Scientific Committee guidance (EFSA Scientific Committee, 2011) and complied with the principles of Good Laboratory Practice (GLP) with some deviations not impacting the study results and interpretation (i.e. diet preparation and analysis including test item stability, homogeneity and concentration), which are detailed below.

Event-specific PCR analysis confirmed the presence of the event MIR604 in the GM maize grains and diets and excluded the presence of the event in the respective controls. ELISA analyses also confirmed the presence of mCry3A and PMI proteins in the GM maize grains and GM diets. Both GM and control maize grains and diets were analysed for nutrients, antinutrients and potential contaminants (e.g. selected heavy metals, mycotoxins and pesticides). Balanced diets were formulated based on the specifications for CT1 rodent base diet.

The stability of the test and control material was not verified; however, in accordance to product expiration declared by the diet manufacturer, the diets are considered stable for the duration of the treatment. The GMO Panel considered this justification acceptable. Diet preparation procedures and regular evaluations of the mixing methods[18] guaranteed the homogeneity and the proper concentration of the test or control substances in them. The applicant provided information on concentration of proximates, fibre, minerals and vitamins in the formulated diets used in the study, further supporting the homogeneity of the formulations.

Feed and water were provided ad libitum. In-life procedures and observations and terminal procedures were conducted in accordance to OECD test guideline 408 (OECD, 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 a diet containing maize MIR604 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:

[18] Including Sodium dispersion testing (appendix 2 Study Report).

- were within the normal variation[19] 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.

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. Increased kidney mineralization was noted in females given MIR604 diet (7/16) as compared to concurrent controls (3/16), but these animals had no indications of altered blood or urinary changes associated with impaired renal function and the finding is not statistically significant ($p = 0.25$, Fisher exact test). Overall, it is concluded that maize MIR604 did not have an adverse effect on the rat kidney.

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 including 50% grains from maize MIR604 for 90 days.

3.4.3.4. Allergenicity

For the allergenicity assessment, a weight-of-evidence approach was followed, taking into account all the information obtained on the newly expressed proteins, as no single piece of information or experimental method yields sufficient evidence to predict allergenicity and adjuvanticity (Codex Alimentarius, 2009; EFSA GMO Panel, 2011a; Commission Regulation (EU) No 503/2013). Furthermore, an assessment of specific newly expressed proteins in relation to their potential to cause celiac disease was also performed (EFSA GMO Panel, 2017b).

Assessment of allergenicity of the newly expressed proteins[20]

The GMO Panel has previously evaluated the safety of Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, CP4 EPSPS (including its variant CP4 EPSPS L214P), mCry3 and PMI proteins individually, and no evidence of allergenicity was identified in the context of the applications assessed (Table 2). No new information on allergenicity of the proteins newly expressed in this four-event stack maize that might change the previous conclusions of the GMO Panel has become available. Based on the current knowledge, and as there is no evidence of allergenicity of the newly expressed proteins, there are no expected concerns of allergenicity as a consequence of their interaction in this four-event stack maize.

The GMO Panel has previously evaluated the safety of the newly expressed proteins, and no evidence of adjuvanticity were identified in the context of the applications assessed (Table 2). This aspect has been discussed in detail by EFSA (2018) and Parenti et al. (2019). To date, there is no evidence for adjuvanticity in the GMOs assessed by the Panel. This four-event stack maize has similar levels of the individual Bt proteins as those in the respective single maize events (see Section 3.4.1.4). The GMO Panel did not find indications that the Bt proteins at the levels expressed in this four-event stack maize might be adjuvants able to enhance an allergic reaction.

The applicant also provided information on the safety of the Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, CP4 EPSPS (including its variant CP4 EPSPS L214P), mCry3 and PMI proteins regarding their potential to cause a celiac disease response. For such assessment, the applicant followed the principles described in the EFSA GMO Panel guidance document (EFSA GMO Panel, 2017b). The assessment of the Cry34Ab1, Cry1Ab, CP4 EPSPS (including its variant CP4 EPSPS L214P) and mCry3 proteins identified no perfect or relevant partial matches with known celiac disease peptide sequences. The assessment of the Cry1F, Cry35Ab1, PAT and PMI proteins revealed partial matches containing the Q/E-X1-P-X2 motif and required further investigations. Several of these partial matches have been previously assessed by the EFSA GMO Panel (2019a,c, 2020 and 2021d,e). Based on additional considerations on position and nature of amino acids flanking the motifs, such as the presence of two

[19] 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 'adverse' 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 standardized 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).

[20] Dossier: Part II – Section 1.5.1 and 1.5.3; additional information 17/6/2019 and 5/8/2020.

consecutive prolines and the charge and size of adjacent amino acids (EFSA GMO Panel, 2017b), the relevant peptides containing the motif do not raise concern as they fail to mimic gluten sequences. Therefore, no indications of safety concern were identified by the GMO Panel.

Assessment of allergenicity of the whole GM plant[21]

The GMO Panel regularly reviews the available publications on food allergy to maize. However, maize is not considered a common allergenic food[22] (OECD, 2002). Therefore, the GMO Panel does not request experimental data to analyse the allergen repertoire of GM maize. In the context of this application and considering the data from the molecular characterisation, the compositional analysis and the assessment of the newly expressed proteins (see Sections 3.4.1, 3.4.2 and 3.4.3), the GMO Panel identifies no indications of a potentially increased allergenicity of food and feed derived from this four-event stack maize with respect to that derived from the comparator and the non-GM reference varieties tested.

3.4.3.5. Dietary exposure assessment to new constituents

In line with Regulation (EU) No 503/2013 the applicant provided dietary exposure estimates to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI and CP4 EPSPS proteins newly expressed in DP4114 × MON 810 × MIR604 × NK603 maize. Dietary exposure was estimated based on protein expression levels reported in this application for the four-event stack maize treated with the intended herbicides, the current available consumption data and feed practices, the foods and feeds currently available in the market and the described processing conditions.

For the purpose of estimating dietary exposure, the levels of newly expressed proteins in DP4114 × MON 810 × MIR604 × NK603 maize grains, forage and pollen were derived from replicated field trials (four replicates from four locations) in the 2015 US growing season (see Section 3.4.1.3). When for a particular newly expressed protein all the data were below the limit of quantification (LOQ), this LOQ was used as expression value to estimate dietary exposure. Table 8 describes the protein expression levels used to estimate both human and animal dietary exposure.

Table 8: Mean values (n = 16, μg/g dry weight and μg/g fresh weight) for newly expressed proteins in grains, forage and pollen from DP4114 × MON 810 × MIR604 × NK603 maize treated with the intended herbicides[(a)]

Protein	Tissue/developmental stage		
	Grains/R6 (μg/g dry weight per fresh weight)	Pollen/R1 (μg/g dry weight)[(b)]	Forage/R4 (μg/g dry weight)
Cry1F	2.9/2.3	42	11
Cry34Ab1	35/27	24	110
Cry35Ab1	0.60/0.48	< LOQ[(c)]	30
PAT	< LOQ[(c)]	< LOQ[(c)]	2.5
Cry1Ab	0.31/0.24	< LOQ[(c)]	13
mCry3A	0.35/0.27	< LOQ[(c)]	15
PMI	1.5/1.2	44	9.9
CP4 EPSPS	15/12	330	130

(a): Protein expression values not corrected for extraction efficiency (see main text for further details). Treated with the intended glyphosate- and glufosinate-ammonium-containing herbicides.

(b): Concentrations values in pollen were adjusted to 6% moisture content before using them to estimate dietary exposure to the different newly expressed protein via the consumption of pollen supplements.

(c): All samples were below the limit of quantification: for PAT protein in grain (LOQ = 0.054 μg/g dry weight/0.043 μg/g fresh weight), for PAT protein in pollen (LOQ = 0.22 μg/g dry weight), for Cry35Ab1 protein in pollen (LOQ = 0.32 μg/g dry weight), for Cry1Ab protein in pollen (LOQ = 0.13 μg/g dry weight), mCry3A protein in pollen (LOQ = 0.28 μg/g dry weight).

[21] Dossier: Part II – Section 1.5.2.

[22] 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.

Human dietary exposure[23]

Chronic and acute dietary exposure to the newly expressed proteins Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI and CP4 EPSPS in DP4114 × MON 810 × MIR604 × NK603 maize grains were provided by the applicant following the methodology described by EFSA to estimate dietary exposure in average and high consumers using summary statistics of consumption (EFSA, 2019a).

Human dietary exposure was estimated across different 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 DP4114 × MON 810 × MIR604 × NK603 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).[24] Corn oil, corn starch and corn syrup were excluded from the assessment since no proteins are expected to be present in these commodities.

Mean protein expression values on fresh weight basis are considered as the most adequate to estimate human dietary exposure (both acute and chronic) when working with raw primary commodities that are commonly consumed as processed blended commodities (EFSA, 2019a). Different recipes and factors were considered to estimate the amount of maize in the consumed commodities before assigning newly expressed protein levels to the relevant commodities.[25] No losses in the newly expressed proteins during processing were considered, except for certain commodities excluded from the exposure estimations (maize oil, corn starch, corn syrup).

The highest acute dietary exposure (high consumers) was estimated in the age class 'Other children' with exposure estimates that ranged between 0.65 mg/kg body weight (bw) per day and 410 mg/kg bw per day for PAT and Cry34Ab1, respectively. The main average contributor to the exposure in the dietary survey with the highest estimates was corn grains.

The highest chronic dietary exposure (high consumers) was estimated in the age class 'Infants' with exposure estimates that ranged between 0.24 and 152 mg/kg bw per day for PAT and Cry34Ab1, respectively. The main average contributor to the exposure in the dietary survey with the highest estimates was sweet corn.

An *ad hoc* dietary exposure scenario was carried out for consumers of pollen supplements under the assumption that these supplements might be made of pollen from DP4114 × MON 810 × MIR604 × NK603 maize. Consumption data on pollen supplements are available for few consumers across eight different European countries.[26] The low number of consumers available adds uncertainty to the exposure estimations which should be carefully interpreted, and it prevents from estimating exposure for high consumers of pollen supplements. In average consumers of pollen supplements, the highest acute dietary exposure would range from 0.09 μg/kg bw per day for Cry1Ab to 230 μg/kg bw per day for CP4 EPSPS, in the elderly population. Similarly, the highest chronic dietary exposure in average consumers would range from 0.06 μg/kg bw per day for Cry1Ab to 153 μg/kg bw per day for CP4 EPSPS, also in the elderly population.

Animal dietary exposure[27]

Dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI and CP4 EPSPS proteins in maize DP4114 × MON 810 × MIR604 × NK603 was estimated across different animal species, as below described, 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 four-event stack maize products was considered.

Mean levels (dry weight) of the newly expressed proteins in grains and forage from the four-event stack maize treated with the intended herbicide used for animal dietary exposure are listed in Table 8. All the grain samples analysed in maize DP4114 × MON 810 × MIR604 × NK603 for the presence of

[23] Dossier: Part II – Section 2, study report PHI-2015-009/010, additional information 05/09/2019.
[24] https://www.efsa.europa.eu/en/applications/gmo/tools. Data accessed July 2019.
[25] 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 24.4 μg of Cry34Ab1 per gram of maize bread as compared to the 27 μg/g reported as mean concentration in the maize grains.
[26] https://www.efsa.europa.eu/en/food-consumption/comprehensive-database. Data accessed August 2021
[27] Additional information 5/9/2019

PAT protein were below the limit of quantification (LOQ = 0.054 μg/g dry weight); for the purpose of estimating dietary exposure, the limit of quantification was used as the assumed mean amount of protein in grain.

The applicant estimated dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI and CP4 EPSPS proteins via the consumption of maize grains in poultry, swine, cattle and sheep, based on default values for animal body weight, daily feed intake and inclusion rates (percentage) of maize feedstuffs in rations, as provided for the EU by OECD (2013). Estimated dietary exposure in the concerned animals is reported in Appendix D.

To further integrate the assessment, the GMO Panel estimated dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI and CP4 EPSPS proteins via the consumption of forage in beef and dairy cows, lamb, breeding swine and layer for forage. Consumption of maize forage is based on default values for animal body weight, daily feed intake and inclusion rates (percentages), as provided for the EU by OECD (2013); estimated dietary exposure in the concerned animals is reported in Appendix D.

3.4.3.6. Nutritional assessment of endogenous constituents

The intended traits of maize DP4114 × MON 810 × MIR604 × NK603 are herbicide tolerance and insect resistance, with no intention to alter nutritional parameters. However, levels of crude protein (treated), carbohydrates (treated) and phosphorus (both treated and not treated) in forage were significantly different from the comparator and showed a lack of equivalence with the set of non-GM reference varieties (Section 3.4.2.6).

Animal nutrition

Forage is an important feed source for herbivores that can utilise it because of the capacity for microbial digestion of cell wall constituents. Forage guarantees the proper function of gastrointestinal tract that is essential for the activity of microbes; moreover, forage alone is able to satisfy nutritional requirements of animals up to a certain level, e.g. low producing dairy cows. Therefore, forage is not provided to animals with the only purpose to fulfil nutritional requirements and the magnitude of the decrease in carbohydrates and increase in crude protein and phosphorus content in maize DP4114 × MON 810 × MIR604 × NK603 forage does not represent a nutritional concern.

3.4.3.7. Conclusion on the food/feed safety assessment

The Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI and CP4 EPSPS proteins newly expressed in maize DP4114 × MON 810 × MIR604 × NK603 do not raise safety concerns for human and animal health. No interactions between the newly expressed proteins relevant for food and feed safety were identified, and no overall toxicological concerns on the four-event stack maize were identified. Similarly, the GMO Panel did not identify indications of safety concerns regarding allergenicity or adjuvanticity related to the presence of the newly expressed proteins in the four-stack maize DP4114 × MON 810 × MIR604 × NK603, or regarding the overall allergenicity of this four-event stack maize. Based on the outcome of the comparative assessment and the nutritional assessment, the GMO Panel concludes that the consumption of maize DP4114 × MON 810 × MIR604 × NK603 does not represent any nutritional concern, in the context of the scope of this application.

3.4.4. Environmental risk assessment[28]

Considering the scope of application EFSA-GMO-NL-2017-150, which excludes cultivation, the environmental risk assessment (ERA) of maize DP4114 × MON 810 × MIR604 × NK603 mainly takes into account: (1) the exposure of microorganisms to recombinant DNA in the gastrointestinal tract of animals fed GM material and of microorganisms present in environments exposed to faecal material of these animals (manure and faeces); and (2) the accidental release into the environment of viable maize DP4114 × MON 810 × MIR604 × NK603 grains during transportation and/or processing (EFSA GMO Panel, 2010a).

[28] Dossier: Part II – Section 5.

3.4.4.1. 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 over winter 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 DP4114 × MON 810 × MIR604 × NK603 and the observed differences in early and final population will provide a selective advantage to maize plants, except when they are exposed to glyphosate- and/or glufosinate-ammonium-containing herbicides or infested by insect pests that are susceptible to the Cry1F, Cry34Ab1, Cry35Ab1, Cry1Ab and/or mCry3A proteins. The GMO Panel considers that the fitness advantage provided by the intended traits, and the observed differences in early and final population (see Section 3.4.2.5) 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 that maize DP4114 × MON 810 × MIR604 × NK603 will be equivalent to 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 DP4114 × MON 810 × MIR604 × NK603 grains.

3.4.4.2. 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

The probability and potential adverse effects of HGT of the recombinant DNA have been assessed in previous GMO Panel Scientific Opinions for the single events (see Table 2). This assessment included consideration of homology-based recombination processes, as well as non-homologous end joining and microhomology-mediated end joining. Possible fitness advantages that the bacteria in the receiving environments would gain from acquiring recombinant DNA were considered. No concern as a result of an unlikely, but theoretically possible, HGT of the recombinant genes to bacteria in the gut of domesticated animals and humans fed GM material or other receiving environments was identified.

The applicant submitted an updated bioinformatic analysis for each of the single events to assess the possibility for HGT by homologous recombination.

The updated bioinformatics analyses of events DP4114, MON 810, MIR604 and NK603 confirm the assessments provided in the context of previous Scientific Opinions (EFSA GMO Panel, 2018a, 2019b, 2021a,f).

Synergistic effects of the recombinant genes, for instance due to combinations of recombinogenic sequences, which would cause an increase in the likelihood for HGT or a selective advantage were not identified.

Therefore, the GMO Panel concludes that the unlikely, but theoretically possible, horizontal transfer of recombinant genes from this number-event stack maize to bacteria does not raise any environmental safety concern.

Plant-to-plant gene transfer

The potential for occasional feral maize DP4114 × MON 810 × MIR604 × NK603 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 and Sweet, 2002; OECD, 2003; EFSA, 2016; 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; Trtikova et al., 2017; Le Corre et al., 2020).

The potential of spilled maize grains to establish, grow and produce pollen is extremely low and transient (see Section 3.4.4.1). 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). 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.

3.4.4.3. Interactions of the GM plant with target organisms

Taking the scope of application EFSA-GMO-NL-2018-150 (no cultivation), potential interactions of occasional feral maize DP4114 × MON 810 × MIR604 × NK603 plants arising from grain import spills with the target organisms are not considered a relevant issue.

3.4.4.4. Interactions of the GM plant with non-target organisms

Given that environmental exposure of non-target organisms to spilled GM grains or occasional feral GM maize plants arising from spilled maize DP4114 × MON 810 × MIR604 × NK603 grains is limited, and because ingested proteins are degraded before entering the environment through faecal material of animals fed GM maize, potential interactions of the four-event stack maize with non-target organisms are not considered by the GMO Panel to raise any environmental safety concern. Interactions that may occur between the Cry proteins will not alter this conclusion.

3.4.4.5. Interactions with abiotic environment and biogeochemical cycles

Given that environmental exposure to spilled grains or occasional feral maize DP4114 × MON 810 × MIR604 × NK603 plants arising from grain import spills is limited, and because ingested proteins are degraded before entering the environment through faecal material of animals fed GM maize, potential interactions with the abiotic environment and biogeochemical cycles are not considered by the GMO Panel to raise any environmental safety concern.

3.4.4.6. Conclusion of the environmental risk assessment

The GMO Panel concludes that it is unlikely that maize DP4114 × MON 810 × MIR604 × NK603 would differ from conventional maize varieties in its ability to persist under European environmental conditions. Considering the scope of application EFSA-GMO-NL-2018-150, interactions of occasional feral maize DP4114 × MON 810 × MIR604 × NK603 plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of HGT from the four-event stack maize to bacteria does not indicate a safety concern. Therefore, considering the combined traits and their interactions, the outcome of the agronomic and phenotypic analysis, and the routes and levels of exposure, the GMO Panel concludes that maize DP4114 × MON 810 × MIR604 × NK603 would not raise safety concerns in the event of accidental release of viable GM maize grains into the environment.

3.5. Risk assessment of the subcombinations

Subcombinations previously assessed in the frame of other applications are discussed in Section 3.5.1. The subcombinations that have not been previously assessed are discussed in Section 3.5.2.

3.5.1. Subcombinations previously assessed

The GMO Panel has previously assessed one subcombination and no safety concerns were identified (see Table 2). Literature searches covering the 10 years before submission of the application and the period since the time of validity of the application revealed no new scientific information relevant to the risk assessment of these maize stacks.[29] Consequently, the GMO Panel considers that its previous conclusions on this subcombination remain valid.

[29] Dossier: Part II – Section 7; additional information: 17/11/2017, 8/1/2018 and 13/7/2020

3.5.2. Subcombinations not previously assessed

Nine of the 10 subcombinations included in the scope of this application have not been previously assessed by the GMO Panel (Table 9). In this case, following the strategy defined by the GMO Panel,[30] the risk assessment takes as its starting point the assessment of the single maize events, and uses the data generated for the four-event stack as well as all the additional data available on subcombinations previously assessed by the GMO Panel (Table 2) and the additional studies provided by the applicant (Appendix A).

Table 9: Maize stacks not previously assessed and covered by the scope of application EFSA-GMO-NL-2018-150

Degree of stacking	Events
Three-event stack	MIR604 × NK603 × DP4114
	MON 810 × NK603 × DP4114
	MON 810 × MIR604 × DP4114
	MON 810 × MIR604 × NK603
Two-event stack	NK603 × DP4114
	MIR604 × DP4114
	MIR604 × NK603
	MON 810 × DP4114
	MON 810 × MIR604

3.5.2.1. Stability of the events

The genetic stability of the inserted DNA over multiple generations in the four single maize events was demonstrated previously (see Table 2). Integrity of the events was demonstrated in the four-event stack maize DP4114 × MON 810 × MIR604 × NK603 (Section 3.4.1.2) and the previously assessed maize subcombination (Table 2). The GMO Panel finds no reasons to expect the loss of integrity of the events in the maize subcombinations not previously assessed (see Table 9).

3.5.2.2. Expression of the events

The GMO Panel assessed whether any combination of the four events by conventional crossing could result in significant changes in expression levels of the newly expressed proteins, as this could indicate an unexpected interaction among the events. Based on current knowledge of the molecular elements introduced, there is no reason to expect interactions that would affect the levels of the newly expressed proteins in the 9 subcombinations compared with those in the single maize events. This assumption was confirmed by comparing the levels of the newly expressed proteins of each single maize event with those of the four-event stack maize. The levels were similar in the four-event stack maize and in the single events (Section 3.4.1.3 and Appendix B). Therefore, there was no indication of an interaction at protein expression level. In addition, expression data from the two-event stack maize MON 810 × NK603 (EFSA, 2005a,b; EFSA GMO Panel, 2017a, 2018b, 2021a) were similar to those observed in each of the single maize events. This supports the conclusion that interactions affecting the expression levels of the newly expressed proteins are not expected in the 9 subcombinations not previously assessed and included in the scope of application EFSA-GMO-NL-2018-150.

3.5.2.3. Potential functional interactions among the events

The GMO Panel assessed the potential for interactions among maize events in the 9 subcombinations not previously assessed (Table 9), taking into consideration intended traits and unintended effects.

Based on the known biological functions of the individual newly expressed proteins (Table 4), there is currently no expectation for possible interactions relevant for the food and feed or environmental safety among these proteins in those subcombinations. The GMO Panel took into account all the intended and potential unintended effects considered in the assessment of the four single events, the previously assessed subcombination (Table 2) and the four-event stack maize. It is concluded that none of these events would raise safety concerns when combined in any of these maize

[30] 115th GMO Panel meeting (Annex 1 of the minutes: http://www.efsa.europa.eu/sites/default/files/event/170517-m.pdf).

subcombinations. The GMO Panel considers that no further data are needed to complete the assessment of subcombinations- from the four-event stack maize.

3.5.3. Conclusion

Since no new safety concerns were identified for the previously assessed subcombination, the GMO Panel considers that its previous conclusions on this maize subcombination remain valid. For the remaining 9 subcombinations included in the scope of application EFSA-GMO-NL-2018-150, the GMO Panel assessed the possibility of interactions among the events and concluded that these combinations would not raise safety concerns. These subcombinations are therefore expected to be as safe as and nutritionally equivalent to the single maize events, the previously assessed subcombination and the four-event stack maize.

3.6. Post-market monitoring

3.6.1. Post-market monitoring of GM food/feed

The GMO Panel concluded that maize DP4114 × MON 810 × MIR604 × NK603, as described in this application, does not raise any nutritional concern and is as safe as the comparator and the non-GM reference varieties tested (Section 3.4.3). One of the subcombinations has been previously assessed and no safety concerns were identified. The subcombinations not previously assessed and included in the scope of this application (nine) are expected to be as safe as the single maize events, the previously assessed maize subcombination and the four-event stack maize (Section 3.5.2). Therefore, the GMO Panel considers that post-market monitoring of food and feed from the four-event stack maize and its subcombinations, as described in this application, is not necessary.

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 the four-event stack maize, no case-specific monitoring is required. The PMEM plan proposed by the applicant for the four-event stack maize includes: (i) 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; (ii) a coordinating system established by EuropaBio for the collection of information recorded by the various operators; and (iii) the review of relevant scientific publications retrieved from literature searches (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a PMEM report on an annual basis and a final report at the end of the authorisation period.

The GMO Panel considers that the scope of the PMEM plan provided by the applicant is consistent with the intended uses of the four-event stack maize. The GMO Panel agrees with the reporting intervals proposed by the applicant in its PMEM plan. The PMEM plan and reporting intervals are in line with the intended uses of the four-event stack maize and its subcombinations.

3.6.3. Conclusions on post-market monitoring

No post market monitoring of food and feed is necessary. The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of the four-event stack maize.

4. Overall conclusions

The GMO Panel was asked to carry out a scientific assessment of maize DP4114 × MON 810 × MIR604 × NK603 and subcombinations for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003.

No new information was identified on the four single maize events (DP4114, MON 810, MIR604 and NK603) that would lead to a modification of the original conclusions on their safety.

The molecular characterisation, the comparative analysis (agronomic, phenotypic and compositional characteristics) and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single maize events and of the newly expressed proteins in the four-event stack maize does not give rise to food/feed safety and nutritional concerns. The GMO Panel concludes that the four-event stack maize, as described in this application, does not raise any nutritional concern and is as safe as its comparator and the selected non-GM reference varieties.

The GMO Panel concludes that there is a very low likelihood of environmental effects resulting from the accidental release of viable grains from the four-event stack maize into the environment. Since no new data were identified on the previously assessed subcombination that would lead to a modification of the original conclusions on their safety, the GMO Panel considers that its previous conclusions on this maize stack remain valid. For the remaining subcombinations included in the scope of application EFSA-GMO-NL-2018-150, no information has been provided. The GMO Panel assessed the possible interactions between the events in these subcombinations and concludes that these combinations of events DP4114, MON 810, MIR604 and NK603 would not raise safety concerns. These subcombinations are therefore expected to be as safe as the maize single events, the previously assessed subcombination and the four-event stack maize.

Based on the relevant publications identified through the literature searches, the GMO Panel did not identify any safety issues pertaining to the intended uses of maize DP4114 × MON 810 × MIR604 × NK603 and its subcombinations.

In addition, the GMO Panel considered the additional unpublished studies listed in Appendix A. This new information does not raise any concern for human and animal health and the environment regarding the four-event stack maize and its subcombinations. Given the absence of safety and nutritional concerns for foods and feeds from the four-event stack maize and all its subcombinations, the GMO Panel considers that PMM of these products is not necessary. The PMEM plan and reporting intervals are in line with the intended uses of the four-event stack maize and its subcombinations. In conclusion, the GMO Panel considers that maize DP4114 × MON 810 × MIR604 × NK603 and its subcombinations, as described in this application, are as safe as the comparator and the selected non-GM reference varieties with respect to potential effects on human and animal health and the environment.

5. Documentation as provided to EFSA

- Application submitted for the authorisation of DP4114 × MON 810 × MIR604 × NK603 maize submitted by Pioneer Overseas Corporation on 8 May 2018 (EFSA Refs. EFSA-GMO-NL-2018-150 – EFSA-Q-2018-00370)
- The application was made valid on 2018-08-10
- Additional Information was Requested on (1) 2018-08-10
- Additional Information was Received on (1) 2018-09-06
- Additional Information was Requested on (2) 2018-09-14
- Additional Information was Received on (2) 2018-11-05
- Additional Information was Requested on (3) 2018-11-07
- Additional Information was Received on (3) 2019-02-08
- Additional Information was Requested on (4) 2018-11-20
- Additional Information was Received on (4) 2019-02-08
- Additional Information was Requested on (5) 2019-03-13
- Additional Information was Received on (5) 2019-06-17
- Additional Information was Requested on (6) 2019-04-12
- Additional Information was Received on (6) 2019-06-11 (partial), 2019-09-05 (partial), 2019-09-23 (complete)
- Additional Information was Requested on (7) 2019-10-29
- Additional Information was Received on (7) 2019-12-17
- Additional Information was Requested on (8) 2019-12-04
- Additional Information was Received on (8) 2020-03-16 (partial) 2020-05-07 (complete)
- Additional Information was Requested on (9) 2019-02-10
- Additional Information was Received on (9) 2020-03-19
- Additional Information was Requested on (10) 2020-05-12

- Additional Information was Received on (10) 2020-08-05
- Additional Information was Requested on (11) 2020-08-25
- Additional Information was Received on (11) 2021-08-23
- Additional Information was Requested on (12) 2021-09-06
- Additional Information was Received on (12) 2021-10-13
- Additional Information was Requested on (13) 2021-10-04
- Additional Information was Received on (13) 2021-12-06
- Additional Information was Requested on (14) 2021-12-22
- Additional Information was Received on (14) 2022-01-14
- Supplementary information was provided on a voluntary basis on 2019-12-17, 2020-01-16 and on 2021-03-23

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Abbreviations

ADF	acid detergent fibre
AOSA	Association of Official Seed Analysts
bw	body weight
CaMV	cauliflower mosaic virus
CRM	comparative relative maturity
CRY	crystal protein
CTP	chloroplast transit peptide
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphat synthase
ERA	environmental risk assessment
GLP	Good Laboratory Practice
GM	genetically modified
GMO Panel	EFSA Panel on Genetically Modified Organisms
GMO	genetically modified organism
HGT	horizontal gene transfer
Hsp	heat shock proteins
LOQ	limit of quantification
NDF	neutral detergent fibre
Nos	nopaline synthase
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PAT	phosphinothricin-acetyl-transferase
PCR	polymerase chain reaction
PMEM	post-market environmental monitoring
PMI	phosphomannose isomerase
SES	standardised effect sizes
TA	*Triticum aestivum*
TDF	total dietary fibre
UTR	untranslated region

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 DP4114 × MON 810 × MIR604 × NK603 for humans, animal or the environment

Study identification	Title
PHI-2011-016	Plot Generation for Maize Lines Containing Events DP-ØØ4114-3, MON-ØØ81Ø-6, SYN-IR6Ø4-5, MON-ØØ6Ø3-6, and the Combined Trait Product DP-ØØ4114-3xMON-ØØ81Ø-6xSYN-IR6Ø4-5xMON-ØØ6Ø3-6: U.S. Test Sites
PHI-2011-017	Agronomic Characteristics and Nutrient Composition of Maize Lines Containing Events DP-ØØ4114-3, MON-ØØ81Ø-6, SYN-IR6Ø4-5, MON-ØØ6Ø3-6, and the Combined Trait Product DP-ØØ4114-3xMON-ØØ81Ø-6xSYN-IR6Ø4-5xMONØØ6Ø3-6: U.S. Test Sites
PHI-2011-120	Expressed Trait Protein Concentration of a Maize Line Containing the Combined Trait Product DP-Ø4114-3xMON-ØØ81Ø-6xSYN-IR6Ø4-5xMON-ØØ6Ø3-6: Chile Test Sites
PHI-2012-032	Agronomic Characteristics, Expressed Trait Protein Concentration, and Nutrient Composition of a Maize Line Containing the Combined Trait Product DP-ØØ4114-3xMON-ØØ81Ø-6xSYN-IR6Ø4-5xMON-ØØ6Ø3-6: U.S. Test Sites
PHI-2012-216	Field Production and Characterization of Grain from a Maize Line Containing the Combined Trait Product DP-ØØ4114-3xMON-ØØ81Ø-6xSYN-IR6Ø4-5xMON-ØØ6Ø3-6: Chile Test Site
PHI-2012-348	Thirteen-Week Rat Study with Maize Grain Containing the Combined Trait Product DP-ØØ4114-3xMON-ØØ81Ø-6xSYN-IR6Ø4-5xMON-ØØ6Ø3-6
PHI-2013-039	Yield of a Maize Line Containing the Combined Trait Product DP-ØØ4114-3xMON-ØØ81Ø-6xSYN-IR6Ø4-5xMON-ØØ6Ø3-6: U.S. Test Sites

Appendix B – Protein expression data

Mean, standard deviation and range of protein levels (ng/mg dry weight) from maize DP4114 × MON 810 × MIR604 × NK603 (not treated) and DP4114, MON 810, MIR604, NK603 (not treated), from field trials performed across four locations in USA in 2015 (n = 16)[(a)].

Protein	Event(s)	Leaf (BBCH16)	Leaf (BBCH19)	Leaf (BBCH63-65)	Root (BBCH16)	Root (BBCH19)	Root (BBCH63-65)	Pollen (BBCH63-65)	Stalk (BBCH63-65)	Whole plant (BBCH63-65)	Forage (BBCH85)	Grain (BBCH87-99)
Cry1F	**DP4114 × MON 810 × MIR604 × NK603**	16[(b)]±10[(c)] (6.7–38)[(d)]	16 ± 13 (8.4–47)	16 ± 6.1 (9.9–29)	10 ± 3.3 (6.7–17)	8.8 ± 2.9 (4.3–17)	7.0 ± 1.2 (5.1–9.1)	47 ± 3.4 (40–50)	8.0 ± 1.3 (5.3–9.4)	11 ± 1.5 (9.5–14)	9.7 ± 1.5 (6.9–13)	2.5 ± 0.59 (1.7–4.0)
	DP4114	16 ± 7.6 (7.1–3.2)	15 ± 12 (6.2–42)	14 ± 6.3 (7.0–27)	8.9 ± 2.9 (4.3–14)	7.9 ± 2.2 (4.3–13)	6.7 ± 2.2 (3.2–11)	46 ± 3.6 (38–51)	6.8 ± 1.2 (5.3–8.6)	12 ± 2.4 (7.4–17)	9.1 ± 1.4 (7.7–14)	2.4 ± 0.54 (1.6–3.6)
Cry34Ab1	**DP4114 × MON 810 × MIR604 × NK603**	33 ± 6.4 (26–47)	42 ± 19 (24–84)	72 ± 15 (44–98)	23 ± 8.0 (10–34)	35 ± 10 (18–51)	44 ± 13 (25–72)	22 ± 3.5 (18–27)	71 ± 17 (43–94)	61 ± 13 (41–93)	87 ± 28 (43–140)	29 ± 7.4 (14–46)
	DP4114	35 ± 7.3 (27–50)	41 ± 21 (26–91)	73 ± 20 (40–100)	27 ± 10 (9.3–41)	31 ± 8.5 (17–42)	45 ± 17 (13–72)	21 ± 3.4 (15–27)	69 ± 19 (37–110)	64 ± 19 (41–99)	100 ± 30 (70–190)	28 ± 4.1 (23–37)
Cry35Ab1	**DP4114 × MON 810 × MIR604 × NK603**	26 ± 7.4 (8.3–37)	45 ± 16 (28–83)	60 ± 11 (47–83)	16 ± 7.4 (8.3–37)	17 ± 6.3 (7.0–27)	12 ± 3.3 (7.0–17)	– (<LOQ)	21 ± 6.1 (11–32)	49 ± 8.6 (38–65)	25 ± 5.5 (14–35)	0.61 ± 0.17 (0.33–0.86)
	DP4114	28 ± 7.6 (16–39)	45 ± 20 (23–97)	61 ± 15 (38–90)	15 ± 7.7 (6.2–29)	17 ± 7.1 (6.2–27)	12 ± 4.5 (5.5–20)	– (< LOQ)	15 ± 2.4 (11–21)	43 ± 7.4 (35–65)	28 ± 7.1 (18–38)	0.50 ± 0.18 (0.30–0.89)
PAT	**DP4114 × MON 810 × MIR604 × NK603**	11 ± 6.3 (4.7–26)	13 ± 5.8 (5.0–23)	14 ± 4.5 (7.7–21)	0.70 ± 0.32 (0.26–1.1)	0.66 ± 0.22 (0.11–1.0)	0.59 ± 0.16 (0.31–0.86)	– (< LOQ)	0.29 ± 0.40 (0.084–1.3)	6.5 ± 1.0 (4.8–8.0)	2.5 ± 1.0 (1.1–4.3)	– (< LOQ)
	DP4114	12 ± 6.2 (4.2–27)	12 ± 6.4 (4.5–24)	12 ± 4.1 (5.0–20)	0.71 ± 0.39 (0.11–1.40)	0.68 ± 0.28 (0.17–1.3)	0.61 ± 0.20 (0.24–0.93)	– (< LOQ)	0.13 ± 0.067 (0.07–0.36)	6.1 ± 0.86 (4.8–7.8)	2.4 ± 0.76 (1.5–3.8)	– (< LOQ)
Cry1Ab	**DP4114 × MON 810 × MIR604 × NK603**	59 ± 18 (34–87)	53 ± 24 (19–94)	42 ± 12 (27–60)	24 ± 7.3 (14–35)	22 ± 5.6 (14–29)	24 ± 7.3 (16–42)	– (< LOQ)	15 ± 3.8 (8.8–21)	24 ± 4.1 (20–32)	12 ± 3.3 (7.6–17)	0.31 ± 0.085 (0.15–0.53)
	MON 810	51 ± 19 (27–87)	52 ± 23 (24–94)	42 ± 14 (24–72)	29 ± 9.5 (18–46)	23 ± 5.9 (8.7–30)	25 ± 5.3 (16–35)	– (< LOQ)	14 ± 2.4 (11–20)	23 ± 3.7 (17–29)	12 ± 3.3 (7.3–20)	0.35 ± 0.079 (0.22–0.49)

Protein	Event(s)	Leaf (BBCH16)	Leaf (BBCH19)	Leaf (BBCH63-65)	Root (BBCH16)	Root (BBCH19)	Root (BBCH63-65)	Pollen (BBCH63-65)	Stalk (BBCH63-65)	Whole plant (BBCH63-65)	Forage (BBCH85)	Grain (BBCH87-99)
mCry3A	**DP4114 × MON 810 × MIR604 × NK603**	21 ± 2.9 (18–26)	18 ± 4.5 (11–26)	15 ± 2.5 (11–18)	25 ± 6.9 (15–40)	19 ± 7.2 (8.8–33)	19 ± 5.8 (9.2–28)	– (< LOQ)	5.6 ± 1.5 (2.7–8.7)	9.9 ± 1.4 (6.8–12)	13 ± 2.6 (7.3–18)	0.26 ± 0.090 (0.13–0.48)
	MIR604	21 ± 3.6 (15–29)	20 ± 6.6 (13–34)	15 ± 2.8 (11–20)	25 ± 9.7 (7.1–46)	18 ± 4.9 (13–31)	18 ± 7.1 (4.2–28)	– (< LOQ)	4.6 ± 1.7 (2.3–7.9)	9.1 ± 1.6 (7.3–12)	13 ± 4.6 (7.3–24)	0.18 ± 0.18[(e)] (< LOQ–0.75)
PMI	**DP4114 × MON 810 × MIR604 × NK603**	12 ± 5.6 (4.9–22)	9.6 ± 5.9 (3.9–22)	8.9 ± 2.8 (4.9–15)	10 ± 3.3 (4.5–15)	8.5 ± 3.2 (2.3–18)	5.8 ± 1.9 (3.7–9.0)	57 ± 5.1 (47–67)	6.3 ± 1.5 (3.5–9.1)	8.8 ± 1.9 (5.1–12)	8.1 ± 1.7 (5.1–11)	1.3 ± 0.46[(f)] (< 0.27–2.3)
	MIR604	12 ± 6.1 (4.3–24)	11 ± 7.4 (4.3–28)	8.9 ± 3.2 (4.3–16)	10 ± 3.2 (4.0–15)	8.8 ± 1.9 (4.9–13)	6.6 ± 1.9 (4.1–11)	51 ± 12 (8.5–63)	5.1 ± 1.1 (3.5–7.2)	7.2 ± 1.0 (4.9–9.0)	7.8 ± 2.7 (3.8–12)	1.3 ± 0.58 (0.71–2.7)
CP4 EPSPS	**DP4114 × MON 810 × MIR604 × NK603**	210 ± 78 (100–340)	260 ± 72 (160–430)	220 ± 37 (160–290)	88 ± 23 (61–130)	81 ± 20 (39–110)	71 ± 14 (43–93)	220 ± 39 (140–260)	110 ± 29 (68–200)	170 ± 26 (130–220)	120 ± 30 (73–170)	9.4 ± 2.5 (3.4–14)
	NK603	200 ± 120 (98–560)	200 ± 73 (110–340)	230 ± 53 (140–330)	87 ± 21 (64–130)	79 ± 25 (39–120)	68 ± 23 (36–120)	220 ± 32 (140–260)	84 ± 8.8 (73–100)	160 ± 12 (130–180)	110 ± 28 (66–160)	9.8 ± 3.2 (5.3–16)

LOQ: limit of quantification.
–: Not determined due to all measurements below LOQ.
(a): Number of samples is n = 16 except n = 15 in DP4114 × MON 810 × MIR604 × NK603 root (BBCH63-65) for Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI, and CP4EPSPS; and for CP4EPSPS in NK603 leaf, root, stalk and whole plant at BBCH63-65 growth stage; n = 14 for Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI, CP4 EPSPS in DP4114 × MON 810 × MIR604 × NK603 leaf and root BBCH16 growth stage.
(b): Mean.
(c): Standard deviation.
(d): Range.
(e): Four sample results were below the LLOQ (LLOQ = 0.069 ng/mg dry weight). A value equal to half the LLOQ value was assigned to those samples to calculate the mean and standard deviation.
(f): One sample result was below the LLOQ (LLOQ = 0.27 ng/mg dry weight). A value equal to half the LLOQ value was assigned to those samples to calculate the mean and standard deviation.

Appendix C – Statistical analysis and statistically significant findings in the 90-day toxicity studies in rats on the whole food/feed

C.1 Statistical analysis of the 90-day study on MIR604 rats

The following endpoints were statistically analysed: body weight including cumulative body weight gain, food consumption and food utilisation, haematology, coagulation, clinical chemistry, urinalysis (specific gravity, urine volume and pH only) and thyroid hormones, functional observations (rearing, grooming, foot splay, tail flick, fore grip, hind grip and temperature), motor activity data and organ weights (absolute, relative to terminal body weight and relative to brain weight).

For all continuous endpoints, mean, standard deviation in terms of the standardised effect sizes (SES) of each dose group were reported for each sex, variable, and period or time interval. The main statistical analysis compared rats consuming the test diet with those consuming the control diet, with the cage as the experimental unit. The data for continuous parameters were analysed with analysis of variance (ANOVA) for the two sexes combined (fixed effects: treatment, sex, sex-by-treatment interaction and block-within-sex). In case a significant sex-by-treatment interaction was identified (and for sex-specific endpoints) the results of a sex-specific analysis were considered for the assessment. For each comparison, point estimates and 95% confidence intervals of the SES were reported to aid the assessment. Historical control data (collected no earlier than 5 years prior to start of the study) were used, when appropriate, to assess the results. No missing data were reported (Table C.1).

Table C.1: Statistically significant findings in 90-day study on MIR604 in rats compared to controls

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
Body weight	Reduced 2% at day 29	Low magnitude. Not an adverse effect of treatment
Feed consumption	Reduced 8% in females in week 2. Increased 7% in both sexes combined in week 12	No difference in overall food consumption or utilisation. Within normal variation. Not an adverse effect of treatment
Functional observation batteries	Hindlimb grip (lower in males (23%), higher in females (17%))	Within normal variation. Deficit present in males pre-test. No related changes in fore limb grip strength. Not an adverse effect of treatment
Motor activities	Higher overall number of X-ambulations in males (60%)	Within normal variation. 15% increase pre-test; not seen in females. Not an adverse effect of treatment
	At the 50–55 min interval in animals (males and females combined): Decreases in basic movements, fine movements, X-ambulations and Y-ambulations	Only seen at a single time point. No significant reductions in the total movement counts. Within normal variation. Not an adverse effect of treatment
Haematology – Red blood cell count	Decreased (2%) males and females combined	Low magnitude. Not an adverse effect of treatment
Haematology – MCV, MCH	Increase (3%) in females	Low magnitude. Not an adverse effect of treatment
Haematology – Platelets	Decreased in males (10%)	Low magnitude. Not an adverse effect of treatment
Clinical chemistry – Cholesterol, HDL, LDL	Decreased (7%, 8% and 17% respectively) in males and females combined	Decreases of this magnitude are not adverse in isolation. Within normal variation. Not an adverse effect of treatment

Statistically significant parameter/endpoint	Finding	GMO Panel interpretation
Clinical chemistry – Total protein	Lower (3%) in males	Low magnitude. Not an adverse effect of treatment
Thyroid weight (absolute and relative to body weight)	Increased (8%) in males and females combined	Low magnitude. No associated changes in hormone levels or histopathology. Not an adverse effect of treatment

Appendix D – Animal dietary exposure

Table D.1: Dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI and CP4 EPSPS proteins (mg/kg bw per day) in livestock, based on the consumption of maize grains

	BW (kg)	TDI feed (kg DM/animal)	IR (%)	Cry1F	Cry34Ab1	Cry35Ab1	PAT
Broiler	1.7	0.12	70	0.14	1.73	0.030	0.0027
Layer	1.9	0.13	70	0.14	1.68	0.029	0.0026
Turkey	7	0.50	50	0.10	1.25	0.021	0.0019
Breeding	260	6	70	0.047	0.57	0.0097	0.00087
Finishing	100	3	70	0.061	0.74	0.013	0.0011
Beef	500	12	80	0.056	0.67	0.012	0.0010
Dairy	650	25	30	0.033	0.40	0.0069	0.00062
Ram/ewe	75	2.5	30	0.029	0.35	0.0060	0.00054
Lamb	40	1.7	30	0.037	0.45	0.0077	0.00069

	BW (kg)	TDI feed (kg DM/animal)	IR (%)	Cry1Ab	mCry3A	PMI	CP4 EPSPS
Broiler	1.7	0.12	70	0.015	0.017	0.074	0.74
Layer	1.9	0.13	70	0.015	0.017	0.072	0.72
Turkey	7	0.50	50	0.011	0.013	0.054	0.54
Breeding	260	6	70	0.0050	0.0057	0.024	0.24
Finishing	100	3	70	0.0065	0.0074	0.032	0.32
Beef	500	12	80	0.0060	0.0067	0.029	0.29
Dairy	650	25	30	0.0036	0.0040	0.017	0.17
Ram/ewe	75	2.5	30	0.0031	0.0035	0.015	0.15
Lamb	40	1.7	30	0.0040	0.0045	0.019	0.19

Table D.2: Dietary exposure to Cry1F, Cry34Ab1, Cry35Ab1, PAT, Cry1Ab, mCry3A, PMI, and CP4 EPSPS proteins (mg/kg bw per day) in livestock, based on the consumption of maize forage

	BW (kg)	TDI feed (kg DM/animal)	IR (%)	Cry1F	Cry34Ab1	Cry35Ab1	PAT
Beef	500	12	80	0.211	2.11	0.58	0.05
Dairy	650	25	60	0.254	2.54	0.69	0.06
Lamb	40	1.7	30	0.140	1.40	0.38	0.03
Breeding swine	260	6	20	0.051	0.51	0.14	0.01
Layer	1.9	0.13	10	0.075	0.75	0.21	0.02

	BW (kg)	TDI feed (kg DM/animal)	IR (%)	Cry1Ab	mCry3A	PMI	CP4 EPSPS
Beef	500	12	80	0.250	0.29	0.1901	2.50
Dairy	650	25	60	0.300	0.35	0.2285	3.00
Lamb	40	1.7	30	0.166	0.19	0.1262	1.66
Breeding swine	260	6	20	0.060	0.07	0.0457	0.60
Layer	1.9	0.13	10	0.089	0.10	0.0677	0.89