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Assessment of genetically modified maize MON 87427 × MON 89034 × MIR162 × NK603 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2016-131)

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Abstract

Maize MON 87427 × MON 89034 × MIR162 × NK603 (four-event stack maize) was produced by conventional crossing to combine four single events: MON 87427, MON 89034, MIR162 and NK603. The GMO Panel previously assessed the four single maize events and four of the subcombinations did not identify safety concerns. No new data on the single maize events or the four subcombinations that could lead to modification of the original conclusions on their safety were identified. 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, as described in this application, is as safe as and nutritionally equivalent to its non-GM comparator and the non-GM reference varieties tested. 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 the six maize subcombinations not previously assessed and concludes that these are expected to be as safe as and nutritionally equivalent to the single events, the previously assessed subcombinations and the four-event stack maize. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of the four-event stack maize. Post-market monitoring of food/feed is not considered necessary. The GMO Panel concludes that the four-event stack maize and its subcombinations are as safe as its non-GM comparator and the tested non-GM reference varieties with respect to potential effects on human and animal health and the environment.

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Summary

Following the submission of application EFSA-GMO-NL-2016-131 under Regulation (EC) No 1829/2003 from Monsanto (hereafter referred to as 'the applicant'), the Panel on Genetically Modified Organisms of the European Food Safety Authority (hereafter referred to as the 'GMO Panel') was asked to deliver a scientific opinion on genetically modified (GM) maize MON 87427 × MON 89034 × MIR162 × NK603 (hereafter referred to as 'four-event stack maize') and its subcombinations independently of their origin (referred to hereafter as 'subcombinations'). The scope of application EFSA-GMO-NL-2016-131 is for the placing on the market of maize MON 87427 × MON 89034 × MIR162 × NK603 and all its subcombinations independently of their origin for food and feed uses, import and processing.

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 MON 87427 × MON 89034 × MIR162 × NK603 is assessed in the context of the assessment of the four-event stack maize. The safety of the subcombinations that either have 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 maize, is assessed separately in the present scientific opinion.

The four-event stack maize was produced by conventional crossing to combine the four single events MON 87427 (expressing the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein), MON 89034 (expressing the Cry1A.105 and Cry2Ab2 proteins), MIR162 (expressing the Vip3Aa20 and the phosphomannose isomerase (PMI) protein) and NK603 (expressing the CP4 EPSPS protein and its variant CP4 EPSPS L214P) to confer resistance to certain lepidopteran pests and 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 guidelines for the risk assessment of GM plants and derived food and feed, the environmental risk assessment of GM plants and the post-market environmental monitoring of GM plants. The GMO Panel considered the information submitted in application EFSA-GMO-NL-2016-131, additional information provided by the applicant during the risk assessment, the scientific comments submitted by the Member States and the relevant scientific literature.

The previous assessments of the single events MON 87427, MON 89034, MIR162, NK603 and four of the subcombinations (3 two-event stacks and a three-event stack) provided a basis for the assessment of the four-event stack maize and the remaining six 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 undertaken, 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. An evaluation of environmental impacts and the post-market environmental monitoring (PMEM) plan was also undertaken.

The molecular data establish that the events stacked in maize MON 87427 × MON 89034 × MIR162 × NK603 have retained their integrity. Protein expression analyses showed that the levels of the newly expressed proteins are similar in the four-event stack maize and in the single events, except for the expected difference for the CP4 EPSPS protein levels resulting from the combination of the MON 87427 and NK603 single events, both producing CP4 EPSPS protein in the four-event stack. No indications of interactions that may affect the integrity of the events and the levels of the newly expressed proteins in this four-event stack maize were identified.

The comparative analysis of forage and grain composition and agronomic/phenotypic characteristics identified no differences between the four-event stack maize and the non-GM comparator that required further assessment for food/feed safety or environmental impact.

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 the four-event stack maize, as described in this application, is as safe as and nutritionally equivalent to its non-GM comparator and the non-GM reference varieties tested.

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 the four-event stack maize 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 four previously assessed subcombinations, 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 these maize subcombinations remain valid. For the remaining six subcombinations included in the scope of application EFSA-GMO-NL-2016-131, no experimental data were provided. The GMO Panel assessed the possibility of interactions between the events in the six subcombinations and concludes that these combinations would not raise safety concerns. These subcombinations are therefore expected to be as safe as and nutritionally equivalent to the single events, the previously assessed subcombinations and the four-event stack maize.

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 MON 87427 × MON 89034 × MIR162 × NK603 and its subcombinations. In the context of annual PMEM reports, the applicant should improve future literature searches according to the GMO Panel recommendations given in this scientific opinion.

Given the absence of safety concerns for foods and feeds from maize MON 87427 × MON 89034 × MIR162 × NK603 and its subcombinations, the GMO Panel considers that post-market monitoring of these products is not necessary. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of the four-event stack maize and its subcombinations.

The GMO Panel concludes that maize MON 87427 × MON 89034 × MIR162 × NK603 and its subcombinations, as described in this application, are as safe as the non-GM comparator and the tested non-GM reference varieties with respect to potential effects on human and animal health and the environment.

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1. Introduction

1.1. Background

On 18 February 2016, the European Food Safety Authority (EFSA) received from the Competent Authority of The Netherlands application EFSA-GMO-NL-2016-131 for authorisation of maize MON 87427 × MON 89034 × MIR162 × NK603 (hereafter referred to as 'the four-event stack maize') (Unique Identifier MON-87427-7 × MON-89Ø34-3 × SYN-IR162-4 × MON-ØØ6Ø3-6), submitted by Monsanto Europe S.A. (hereafter referred to as 'the applicant') according to Regulation (EC) No 1829/2003.[1]

Following receipt of application EFSA-GMO-NL-2016-131, EFSA informed the EU Member States and the European Commission and made the summary of the application available to the public on the EFSA website.[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 31 May 2016, EFSA declared the application valid and made the valid application available to EU Member States and the European Commission.

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-2016-131. 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 three months to make their opinion known on application EFSA-GMO-NL-2016-131 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 the four-event stack maize and all its subcombinations independently of their origin, in the context of their scope as defined in application EFSA-GMO-NL-2016-131.

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, including the opinions of the nominated risk assessment bodies of EU Member States.[5]

In addition to the present scientific opinion on maize MON 87427 × MON89034 × MIR162 × NK603, EFSA and its GMO Panel were also asked to report on the particulars listed under Articles 6(5) and 18(5) of Regulation (EC) No 1829/2003. The relevant information is made available in the EFSA Register of Questions,[6] including: the information required under Annex II to the Cartagena Protocol, a labelling proposal and a post-market environmental Monitoring (PMEM) plan as provided by the applicant; the method(s), validated by the Community reference laboratory, for detection, including sampling and identification of the transformation event in the food-feed and/or foods-feeds produced from it; and the appropriate reference materials.

[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] Available online at the EFSA Register of Questions (http://registerofquestions.efsa.europa.eu/roqFrontend/questionDocumentsLoader?question=EFSA-Q-2016-00148).

[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] Opinions of the nominated risk assessment bodies of EU Member States can be found at the EFSA Register of Questions (http://registerofquestions.efsa.europa.eu/roqFrontend/questionDocumentsLoader?question=EFSA-Q-2016-00148).

[6] http://registerofquestions.efsa.europa.eu/roqFrontend/questionDocumentsLoader?question=EFSA-Q-2016-00148

2. Data and methodologies

2.1. Data

The GMO Panel based its scientific assessment of the four-event stack maize and subcombinations on application EFSA-GMO-NL-2016-131, additional information provided by the applicant during the risk assessment, scientific comments submitted by 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 provisions of Regulation (EU) No 503/2013. A list of these additional unpublished studies is provided in Appendix B.

2.2. Methodologies

The GMO Panel conducted its assessment in line with the principles described in Regulation (EU) No 503/2013, its applicable guidelines (EFSA GMO Panel, 2010a, 2011a,b, 2015a) and explanatory notes (EFSA, 2017a,b) for the risk assessment of GM plants. During its risk assessment, the GMO Panel considered all additional unpublished studies as listed in Appendix B for potential effects on human and animal health and the environment.

For the assessment of 90-day animal feeding studies, the GMO Panel took into account the criteria included in the EFSA guidance (EFSA Scientific Committee, 2011) and the explanatory statement for its applicability (EFSA, 2014).

The GMO Panel also assessed the applicant's literature searches, which include a scoping review, in accordance with the recommendations on literature searching outlined in EFSA (2010, 2017a).

In the frame of the contracts OC/EFSA/GMO/2013/01 and OC/EFSA/GMO/2014/01, contractors performed preparatory work and delivered reports on the methods applied by the applicant in performing bioinformatic and statistical analyses, respectively.

3. Assessment

3.1. Introduction

Application EFSA-GMO-NL-2016-131 covers the four-event stack maize MON 87427 × MON 89034 × MIR162 × NK603 and all its 10 subcombinations independently of their origin (Table 1). The scope of this application is for food and feed uses, import and processing, and excludes cultivation within the European Union (EU).

Table 1: Stacked maize events covered by the scope of application EFSA-GMO-NL-2016-131

Degree of Stacking	Events
Four-event stack maize	MON 87427 × MON 89034 × MIR162 × NK603
Three-event stack maize	MON 87427 × MON 89034 × MIR162
	MON 87427 × MON 89034 × NK603
	MON 87427 × MIR162 × NK603
	MON 89034 × MIR162 × NK603
Two-event stack maize	MON 87427 × MON 89034
	MON 87427 × NK603
	MON 87427 × MIR162
	MON 89034 × NK603
	MON 89034 × MIR162
	MIR162 × NK603

The term 'subcombination' refers to any combination of up to three of the maize events MON 87427, MON 89034, MIR162 or NK603.

The safety of subcombinations occurring as segregating progeny in harvested grains of the four-event stack maize is evaluated in the context of the assessment of the four-event stack maize in Section 3.4 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: MON 87427 (expressing the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein); MON 89034 (expressing the Cry1A.105 and Cry2Ab2 proteins); MIR162 (expressing the Vip3Aa20 and phosphomannose isomerase (PMI) proteins); and NK603 (expressing the CP4 EPSPS protein and the variant CP4 EPSPS L214P) to confer resistance to certain lepidopteran pests and tolerance to glyphosate-containing herbicides. It should be noted that the assessment of herbicide residues in maize herbicide-tolerant crops relevant for this application has been investigated by the EFSA Pesticides Unit (EFSA, 2018).

All four single maize events, the two-event stacks MON 89034 × NK603, MON 87427 × NK603 and MON 87427 × MON 89034 and the three-event stack MON 87427 × MON 89034 × NK603 have been previously assessed by the GMO Panel (see Table 2), and no safety concerns were identified.

Table 2: Single maize events and subcombinations of maize MON 87427 × MON 89034 × MIR162 × NK603 previously assessed by the GMO Panel

Event	Application or mandate	EFSA Scientific Opinion
MON 87427	EFSA-GMO-BE-2012-110	EFSA GMO Panel (2015b)
MON 89034	EFSA-GMO-NL-2007-37	EFSA (2008)
MIR162	EFSA-GMO-DE-2010-82	EFSA GMO Panel (2012)
NK603	CE/ES/00/01 EFSA-GMO-NL-2005-22 EFSA-GMO-RX-NK603	EFSA (2004, 2007) EFSA (2009) EFSA (2009)
MON 89034 × NK603	EFSA-GMO-NL-2007-38	EFSA GMO Panel (2009)
MON 87427 × MON 89034	EFSA-GMO-BE-2013-117	EFSA GMO Panel (2017a)
MON 87427 × NK603	EFSA-GMO-BE-2013-117	EFSA GMO Panel (2017a)
MON 87427 × MON 89034 × NK603	EFSA-GMO-BE-2013-117	EFSA GMO Panel (2017a)

3.2. Updated information on the single events[7]

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

Updated bioinformatic analyses for maize events MON 87427, MON 89034, MIR162 and NK603 confirmed that no known endogenous genes were disrupted by any of the inserts.

Updated bioinformatic analyses of the amino acid sequence of the newly expressed CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20 and PMI proteins confirm previous results indicating no significant similarities to toxins or allergens[7]. Updated bioinformatics analyses of the newly created open reading frames (ORFs) within the inserts or spanning the junctions between the insert and the flanking regions for maize events MON 87427, MON 89034, MIR162 and NK603 confirmed previous analyses (Table 2). These analyses indicate that the expression of an ORF showing significant similarities to toxins or allergens for any of the events in maize MON 87427 × MON 89034 × MIR162 × NK603 is highly unlikely (Table 2).

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination (HR), the applicant performed a sequence identity analysis to microbial DNA for maize events MON 87427, MON 89034, MIR162 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 EFSA GMO Panel considers that its previous conclusions on the safety of the single maize events remain valid.

3.3. Systematic literature review[8]

The GMO Panel assessed the applicant's literature searches on maize MON 87427 × MON 89034 × MIR162 × NK603, which include a scoping review, according to the guidelines given in EFSA (2010).

A systematic review as referred to in Regulation (EU) No 503/2013 has not been provided in support to the risk assessment of the application EFSA-GMO-NL-2016-131. Based on the outcome of

[7] Dossier: Part II – Sections 1.2.2.2 and 1.2.2.5; additional information: 11/7/2017, 23/11/2018 and 22/3/2019.
[8] Dossier: Part II – Section 7; additional information: 18/7/2017, 14/9/2017, 25/9/2017, 20/12/2017, 30/11/2018 and 25/3/2019.

the scoping review, the GMO Panel agrees that there is limited value of undertaking a systematic review for maize MON 87427 × MON 89034 × MIR162 × NK603 at present.

Although the overall quality of the performed literature searches is acceptable, the GMO Panel considers that future searches on maize MON 87427 × MON 89034 × MIR162 × NK603 should be improved. The GMO Panel therefore recommends the applicant to:

- ensure that enough search term variation is used (covering possible synonyms, related terms, acronyms, spelling variants, old and new terminology, brand and generic names, lay and scientific terminology, common typos, translation issues);
- use truncation consistently.

None of the relevant publications identified through the literature searches reported information pointing to safety issues associated with the intended uses of maize MON 87427 × MON 89034 × MIR162 × NK603.

3.4. Risk assessment of the four-event stack maize MON 87427 × MON 89034 × MIR162 × NK603

3.4.1. Molecular characterisation[9]

In line with the requirements laid down by Regulation (EU) No 503/2013, the possible impact of the combination of the events on their integrity, 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 their biological function

Maize events MON 87427, MON 89034, MIR162 and NK603 were combined by conventional crossing to produce maize MON 87427 × MON 89034 × MIR162 × NK603. The structure of the inserts introduced into the four-event stack maize is 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 MON 87427 × MON 89034 × MIR162 × 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 Cry1A.105, Cry2Ab2 and Vip3Aa20 proteins in susceptible insects.

Table 3: Genetic elements in the expression cassettes of events stacked in maize MON 87427 × MON 89034 × MIR162 × NK603

Event	Promoter	5′ UTR	Transit peptide	Coding region	Terminator
MON 87427	35S (CaMV)	–	CTP2 (*Arabidopsis thaliana*)	CP4 *epsps** (*Agrobacterium* sp.)	*nos* (*Agrobacterium tumefaciens*)
MON 89034	35S (CaMV)	*cab* (*Triticum* sp.)	–	*cry1A.105* (*Bacillus thuringiensis*)	*hsp17* (*Triticum* sp.)
	35S (FMV)	–	CTP (*Zea mays*)	*cry2Ab2* (*B. thuringiensis*)	*nos* (*A. tumefaciens*)
MIR162	*ZmUbiInt* (*Z. mays*)	–	–	*vip3Aa20* (*B. thuringiensis*)	*35S* (CaMV)
	ZmUbiInt (*Z. mays*)	–	–	*pmi* (*Escherichia coli*)	*nos* (*A. tumefaciens*)
NK603	*ract1* (*Oryza sativa*)	*ract1* (*O. sativa*)	CTP2 (*A. thaliana*)	CP4 *epsps** (*Agrobacterium* sp.)	*nos* (*A. tumefaciens*)
	35S (CaMV)	I-*Hsp70* (*Z. mays*)	CTP2 (*A. thaliana*)	CP4 *epsps* L214P (*Agrobacterium* sp.)	*nos* (*A. tumefaciens*)

CaMV: cauliflower mosaic virus; FMV: Figwort Mosaic Virus; CTP: chloroplast transit peptide; UTR: untranslated region.
–: When no element was specifically introduced to optimise expression.
*: Codon-optimised for expression in plants.

[9] Dossier: Part II – Section 1.2.2; additional information: 6/7/2016 and 10/1/2017.

Table 4: Characteristics and intended effects of the events stacked in maize MON 87427 × MON 89034 × MIR162 × NK603

Event	Protein	Donor organism and biological function	Intended effects in GM plant
MON 87427	CP4 EPSPS	Based on a gene from *Agrobacterium* strain CP4 (Barry et al., 2001). 5-enolpyruvyl-shikimate-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 MON 87427 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
MON 89034	Cry1A.105	Based on genes from *Bacillus thuringiensis* subsp. *kurstaki* and 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)	Event MON 89034 expresses a modified version of the Cry1A-type protein. Cry1A.105 is a protein toxic to certain lepidopteran larvae feeding on maize
	Cry2Ab2	Based on a gene from *Bacillus thuringiensis* subsp. *kurstaki*. *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)	Event MON 89034 expresses the Cry2Ab2, a protein toxic to certain lepidopteran larvae feeding on maize
MIR162	Vip3Aa20	Based on a gene from *Bacillus thuringiensis* strain AB88 (Estruch et al., 1996). In addition to Cry proteins, *B. thuringiensis* also produces insecticidal proteins during its vegetative growth stage. These are referred to as vegetative insecticidal proteins (Vip) (Fang et al., 2007)	Event MIR162 expresses a modified version of the *B. thuringiensis vip3Aa1* gene, and encodes Vip3Aa20, a protein toxic to certain lepidopteran larvae feeding on maize
	PMI	Based on a gene from *E. coli*. The phosphomannose isomerase (PMI) enzyme catalyzes the isomerisation of mannose-6-phosphate to fructose-6-phosphate and plays a role in the metabolism of mannose (Markovitz et al., 1967)	Event MIR162 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 a modified version of the bacterial CP4 EPSPS protein which confers tolerance to glyphosate-containing herbicides as it has lower affinity towards glyphosate than the plant endogenous enzyme

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

The genetic stability of the inserted DNA over multiple generations in the single maize events MON 87427, MON 89034, MIR162 and NK603 was previously demonstrated (see Table 2). Integrity of these genetically independent events in maize MON 87427 × MON 89034 × MIR162 × NK603 was demonstrated by polymerase chain reaction (PCR) and sequence analysis, which show that the sequences of the events (inserts and their flanking regions) in the four-event stack maize are identical to the sequences originally reported for the four single events.

3.4.1.3. Information on the expression of the inserts

CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20 and PMI protein levels were analysed by enzyme-linked immunosorbent assay (ELISA) in material harvested from a field trial at five locations in the USA in 2013. Samples analysed included leaf (V2–V4), whole plant (V10–V11), root (V2–V4 and R5), forage (R5), pollen (R1) and grain (R6) both those treated and not treated with glyphosate.

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 maize were comparable to those of the single events, except for the expected differences in the CP4 EPSPS protein levels resulting from the combination of single events MON 87427 and NK603 both producing CP4 EPSPS protein in the four-event stack maize (Appendix A). Therefore, there is no indication of interactions that may affect the levels of the newly expressed proteins in this stack.

3.4.1.4. Conclusions of the molecular characterisation

The molecular data establish that the events stacked in maize MON 87427 × MON 89034 × MIR162 × NK603 have retained their integrity. Protein expression analyses showed that the levels of the newly expressed proteins are comparable in the four-event stack and in the single events except for CP4 EPSPS, which showed in general the expected higher levels in the stack resulting from the combination of the single events MON 87427 and NK603. Therefore, there is no indication of interaction that may affect the integrity of the events or the levels of the newly expressed proteins in this stack.

Based on the known biological function of the newly expressed proteins, the only foreseen interactions at the biological level are between the Cry and Vip3Aa20 proteins in susceptible insects, which will be dealt with in Section 3.4.4.

3.4.2. Comparative analysis[10]

3.4.2.1. Overview of studies conducted for the comparative analysis

Application EFSA-GMO-NL-2016-131 presents data on agronomic and phenotypic characteristics, as well as on forage and grain composition of the four-event stack maize (Table 5).

Table 5: Overview of the comparative analysis studies to characterise the four-event stack maize provided in application EFSA-GMO-NL-2016-131

Study focus	Study details	Comparator	Non-GM reference varieties
Agronomic and phenotypic analysis	Field study, USA, 2013, eight sites[(a)]	MPA640B	17 [(b)]
Compositional analysis			

(a): The field trials were located in Jefferson, IA; Stark, IL; Shelby IL; Clinton IN; Polk, NE; York, NE; Lehigh, PA and Walworth, WI.

(b): Non-GM maize hybrids used in the 2013 field trials (for both the agronomic/phenotypic and the compositional analysis): Burrus 645, Dekalb DKC59-34, Dekalb DKC63-43, Gateway 4148, Gateway 6158, H-9180, Legacy L7671, Lewis 6442, Lewis 7007, LG2597, Midland Phillips 7B15P, Mycogen 2M746, NC + 5220, Phillips 717, Stewart S588, Stewart S602 and Stine 9724.

[10] Dossier: Part II – Section 1.3; additional information: 29/9/2016 and 21/4/2017.

3.4.2.2. Experimental field trial design and statistical analysis

At each site, the following materials were grown: the four-event stack maize, the comparator maize MPA640B and four commercial non-GM maize reference varieties (henceforth, non-GM reference varieties). All materials were treated with conventional herbicides management regimes; in addition, the field trials included the four-event stack maize exposed to the intended glyphosate-containing herbicide on top of the conventional herbicides.

The agronomic, phenotypic and compositional data were analysed as specified by EFSA GMO Panel (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 stack maize and its non-GM comparator) and an equivalence test (between the GM stack maize and the set of non-GM reference varieties).[11] The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).[12]

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 MON 87427, MON 89034, MIR162 and NK603 were transferred in the genetic background of two different non-GM maize inbred lines, LH244 and LH287.

In subsequent subsections, maize MON 87427 × MON 89034 × MIR162 × NK603 refers to the hybrid (F_1) obtained crossing the GM inbred line LH244 (carrying MIR162) with the GM inbred line LH287 (carrying MON 87427 × MON 89034 × NK603).

The comparator selected in the field trials is the hybrid maize MPA640B that was obtained by crossing the non-GM inbred lines LH244 and LH287. As documented by the pedigree and by the requested additional information, the EFSA GMO Panel considers the selected comparator acceptable for the comparative analysis.

The four-event stack maize and the non-GM comparator, both with a comparative relative maturity (CRM) of 110, are appropriate for growing in a range of environments across North America.

Selection of non-GM reference varieties

The 17 non-GM reference varieties (see Table 5) with a CRM ranging from 109 to 115 were selected by the applicant and at each field trial site four of them were tested. On the basis of the information provided on the CRM, the GMO Panel considers the selected non-GM reference varieties appropriate for the comparative assessment.

Seed production and quality

The seeds of the four-event stack maize and the comparator used in the 2013 field trials (see Table 5) were produced, harvested and stored under similar conditions. The seed lots were verified for their identity via event specific PCR analysis. No indications of possible differences in germination capacity (early stand count) were identified under open field conditions (see Section 3.4.2.5). The GMO Panel considers that the starting seed used as test material in the agronomic, phenotypic and compositional studies were of acceptable quality.

Conclusion on suitability

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

[11] The purpose of the test of equivalence is to evaluate the estimated mean values for maize MON 87427 × MON 89034 × MIR162 × NK603 taking into account natural variability as defined by a set of commercial non-GM maize reference varieties with a history of safe use for consumption as food or feed.

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

3.4.2.4. Representativeness of the receiving environments

Selection of field trial sites

The selected field trial sites were located in commercial maize-growing regions of North America. The soil characteristics of the selected fields were diverse,[13] corresponding to optimal and near-optimal conditions for maize cultivation (Sys et al., 1993). The GMO Panel considers that the selected sites 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 monthly basis. An exceptional weather condition was reported at one of the selected site.[14] However, due to the lack of major impacts on plant growth at this site, 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 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, plus exposed to the intended glyphosate-containing herbicide. Glyphosate was applied at the V2–V4 growth stage. The GMO Panel considers that the management practices, including sowing, harvesting and application of plant protection products, were appropriate.

Conclusion on representativeness

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

3.4.2.5. Agronomic and phenotypic characteristics

Thirteen agronomic and phenotypic endpoints,[15] plus information on abiotic stressors, disease incidence and arthropod damage, were collected from eight different sites (Table 5).

The results of the statistical analysis (Section 3.4.2.2) were the following:

- For maize MON 87427 × MON 89034 × MIR162 × NK603 (treated with conventional herbicides), the test of difference identified statistically significant differences with the non-GM comparator for days to 50% pollen shed, ear height, test weight and yield. All the four endpoints fell under equivalence category I.
- For maize MON 87427 × MON 89034 × MIR162 × NK603 (treated with the intended herbicide), a statistically significant difference with the non-GM comparator was identified for test weight, which fell under equivalence category I.

3.4.2.6. Compositional analysis

Maize forage and grains harvested from the field trials in the USA in 2013 were analysed for 78 different constituents (9 in forage and 69 in grains), including the key constituents recommended by the OECD (2002). Fifteen grain constituents with more than 50% of the observations below the limit of quantification were excluded from the statistical analysis.[16]

[13] Soil types of the field trials were silty clay loam, silt loam and loam. Soil organic matter ranged from 2.0% to 3.4%.
[14] Frost event occurred prior to harvest at Walworth County, Wisconsin.
[15] Early stand count, days to 50% pollen shed, days to 50% silking, stay green rating, ear height, plant height, dropped ears, stalk lodged plants, root lodged plants, final stand count, grain moisture, test weight and yield.
[16] These were: sodium, furfural, caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1),), heptadecenoic acid (C17:1), heptadecadienoic acid (C17:2), γ-linolenic acid (C18:3), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3) and arachidonic acid (C20:4).

The statistical analysis (Section 3.4.2.2) was applied to the remaining 63 constituents (9 in forage[17] and 54 in grains[18]). A summary of the outcome of the test of difference and the test of equivalence is presented in Table 6:

- For maize MON 87427 × MON 89034 × MIR162 × NK603 (untreated), the test of difference identified statistically significant differences from the non-GM comparator for 30 constituents (3 in forage and 27 in grains). The level of all 30 constituents fell under equivalence category I or II.
- For maize MON 87427 × MON 89034 × MIR162 × NK603 (treated), statistically significant differences between the stacked maize event and the non-GM comparator were identified for 28 constituents (2 in forage and 26 in grains). The level of all 28 constituents fell under equivalence category I or II.

Table 6: Outcome of the comparative compositional analysis in grains and forage of maize MON 87427 × MON 89034 × MIR162 × 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	31	30[(d)]	33	28[(d)]
	Category III/IV	1[(e)]	_[(f)]	1[(e)]	_[(f)]
	Not categorised	1[(g)]	_[(h)]	1[(g)]	_[(h)]
	Total endpoints	63		63	

(a): Comparison between the four-event stack maize and the non-GM 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/not treated with the intended herbicide glyphosate (see Section 3.4.2.4).
(d): Endpoints with significant differences between the four-event stack maize and its non-GM comparator and falling in equivalence category I-II. For grain, both treated and not treated: alanine, phytic acid, raffinose, arachidic acid (C20:0), eicosenoic acid (C20:1), behenic acid (C22:0), TDF, iron, manganese, phosphorus, potassium, zinc, ash, carbohydrates, protein, total fat, ferulic acid, ß-carotene, pyridoxine and α-tocopherol. For not treated only: threonine, palmitic acid (C16:0), calcium, copper, magnesium, folic acid and riboflavin. For treated only: isoleucine, methionine, stearic acid (C18:0), linolenic acid (C18:3), ADF and thiamine. For forage, both treated and not treated: calcium and moisture. For not treated only: phosphorus.
(e): Endpoints with no significant differences between the four-event stack maize and its non-GM comparator and falling in equivalence category III/IV: palmitoleic acid (C16:1) in grain.
(f): Endpoints with significant differences between the four-event stack maize and its non-GM comparator and falling in equivalence category III/IV: none.
(g): Endpoints not categorised for equivalence and without significant differences between the four-event stack maize and its non-GM comparator: serine in grain.
(h): Endpoints not categorised for equivalence and with significant differences between the four-event stack maize and its non-GM comparator: none.

The GMO Panel assessed all significant differences between the four-event stack maize and the non-GM comparator, taking into account the potential impact on plant metabolism and the natural variability observed for the set of non-GM reference varieties. No endpoints showing significant differences between the four-event stack maize and the non-GM comparator and falling under category III/IV were identified.

[17] Crude protein, crude fat, crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), ash, carbohydrates, calcium and phosphorus.
[18] Proximates and fibre fractions (moisture, ash, protein, total fat, carbohydrates, acid detergent fibre (ADF), neutral detergent fibre (NDF) and total dietary fibre (TDF)), 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), linolenic acid (C18:3), arachidic acid (C20:0), behenic acid (C22:0) and eicosenoic acid (C21:0)), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc), vitamins (folic acid, niacin, pyridoxine, riboflavin, thiamine,β-carotene and α-tocopherol) and other compounds (ferulic acid, *p*-coumaric acid, phytic acid and raffinose).

3.4.2.7. Conclusion on 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 the agronomic and phenotypic characteristics tested between the four-event stack maize and the non-GM comparator needs further assessment for environmental safety.
- None of the differences identified in forage and grain composition between the four-event stack maize and the non-GM comparator needs further assessment for food/feed safety.

3.4.3. Food and feed safety assessment[19]

3.4.3.1. Effects of processing

Maize MON 87427 × MON 89034 × MIR162 × 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. Influence of temperature and pH on newly expressed proteins

The effects of temperature and pH on the newly expressed proteins in this four-event stack maize have been previously evaluated by the GMO Panel (Table 2).

3.4.3.3. Toxicology

Testing newly expressed proteins

Five proteins (Cry1A.105, Cry2Ab, PMI, Vip3Aa20, CP4 EPSPS and its variant CP4 EPSPS L214P) are newly expressed in the four-event stack maize (Section 3.4.1). The GMO Panel has previously assessed these proteins in the context of the single events (Table 2), and no safety concerns were identified for humans and animals. The GMO Panel is not aware of any new information that would change this conclusion.

The potential for a functional interaction between the proteins newly expressed in maize MON 87427 × MON 89034 × MIR162 × NK603 has been assessed with regard to human and animal health. The insecticidal proteins Cry1A.105 and Cry2Ab2 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). The Vip3Aa20 protein is a protein secreted by *Bacillus thuringiensis* during its vegetative phase acting in target insects via a mechanism similar to that of Cry proteins (Chakroun et al., 2016; Bel et al., 2017). The CP4 EPSPS and PMI proteins are enzymes that catalyse distinct biochemical reactions and act on unrelated substrates in the plant with high substrate specificity.

On the basis of the known biological function of the individual newly expressed proteins (Table 4), there is currently no expectation for possible interactions relevant to the food and feed safety of the four-event stack maize.

In vitro protein degradation studies on Cry1A.105, Cry2Ab, PMI, Vip3Aa20, CP4 EPSPS and its variant CP4 EPSPS L214P proteins have been previously evaluated by the GMO Panel (Table 2).

The GMO Panel concludes that there are no safety concerns to human and animal health related to the newly expressed proteins Cry1A.105, Cry2Ab, PMI, Vip3Aa20, CP4 EPSPS and its variant CP4 EPSPS L214P in the four-event stack maize.

Testing of new constituents other than newly expressed proteins

No new constituents other than newly expressed proteins have been identified in the four-event stack maize. Therefore, no further food and feed safety assessment of components other than the newly expressed proteins is required.

[19] Dossier: Part II – Sections 1.4, 1.5, 1.6 and Section 2; additional information: 16/10/2018, 8/11/2018, 22/3/2019 and 26/3/2019.

Information on altered levels of food and feed constituents

The four-event stack maize did not show any compositional differences to the non-GM comparator that would require further assessment (Section 3.4.2.6).

Testing of the whole genetically modified food and feed

Based on the outcome of the molecular characterisation assessment, 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 the four-event stack maize have been identified (see above and Sections 3.4.1 and 3.4.2). Therefore, animal studies on food/feed derived from the 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 MON 87427, MON 89034, MIR162 and NK603. The four studies had already been provided in the context of the single-event applications and assessed by the GMO Panel; no adverse effects related to the administration of the respective GM diets had been identified (Table 2). In the context of the assessment of this four-event stack maize and in order to fulfil the requirements of Regulation (EU) No 503/2013 for 90-day studies, the applicant provided additional information upon EFSA's request: missing information on test material and diets for all the studies; evaluation of the cage effect in the study on MIR162; additional histopathological analysis for the studies on MON 87427 and MON 89034.

These studies are adapted from OECD TG 408 (OECD, 1998) and comply with the principles of Good Laboratory Practice (GLP), except for the lack of analytical determination of concentration, homogeneity and stability of the test item in the formulated diets. It is recognised that it may not always be technically possible to generate information on homogeneity and concentration for a test item administrated or formulated, and the lack of such data and its impact on the validity of a study should be justified (OECD, 2018). The GMO Panel acknowledges that there are no practical methods available to analytically determine these for complex test items such as maize in formulated diets and considers adequate the application of proper diet preparation procedures and regular evaluations of the mixing methods. Based on the additional information received from the applicant the GMO Panel considers that the diet preparation procedures in place in the facilities where the diets for the 90-day studies on MON 87427, MON 89034, MIR162 and NK603 were prepared guaranteed their homogeneity and the proper concentration of the respective test or control items. As regards the stability of the test item (maize grains) in the diets, the applicant considers that in accordance to product expiration standards declared by the diet manufacturer the constituents of the diets used in these studies are stable for the duration of the treatment. The GMO Panel considers this justification acceptable. In addition, the GMO Panel notes that even though the diets were prepared and analysed in non-GLP facilities, standardised procedures and quality measures were followed. Therefore, the GMO Panel considers that this is not a major deviation impacting these studies.

Regarding the 90-day feeding study in rats on MIR162, the applicant also confirmed the identity of the test material and diets and provided grain compositional analysis. The GMO Panel notes that the applicant detected and measured the levels of the newly expressed proteins in maize MIR162 in the test diets after the completion of the 90-day study; the GMO Panel considers that this supports the confirmation of the identity and the stability of the test item and diets. The original statistical analysis (based on individual animals) was complemented by an analysis based on cage mean values for endpoints showing a statistically significant cage effect (between-cage variation larger than within-cage variation).[20] In this additional analysis, no statistically significant differences were identified between rats given the test diets and controls.

Regarding the 90-day studies in rats on MON 89034 and on MON 87427, in the tissues and organs newly examined[21] sporadic histopathological findings were observed and are considered compatible with the spontaneous background pathology of rats of this strain and age.

[20] Body weight (day 7, week 3,7,8,10 and 11); basophil count, eosinophil count, other cells, reticulocytes, landing foot splay, adrenal gland absolute and relative weight, spleen absolute weight.

[21] Aorta, bone (sternum) with bone marrow, cecum, cervix (females only), eyes with optic nerves, lung (including bronchi), mandibular lymph node, Peyer's Patches, skin with mammary gland (females only), skin from males (similar area), oesophagus, pituitary, prostate (males only), mandibular salivary gland, seminal vesicles (males only), skeletal muscle, trachea, urinary bladder, uterus (females only), and vagina (females only) from all animals given the control and 33% test diet.

The GMO Panel noted that the incorporation rate of maize selected in these studies is up to 41.5%, in line with commercially available rodent diets. It has been recently reported that a diet incorporating 50% maize may be tolerated without inducing nutritional imbalances in rats after 90-day administration (Steinberg et al., 2019) but the GMO Panel considers that further scientific confirmation is needed before this 50% maize incorporation rate is generally applicable.

On the basis of the additional information received, the GMO Panel concludes that these studies are in line with the requirements of Regulation (EU) 503/2013 and confirms its previous conclusions, i.e. that there are no indications of adverse effects related to the 90-day administration to rats of diets including grains from maize MON 87427 (33%), MON 89034 (up to 33%) MIR162 (up to 41%) and NK603 (up to 33%).

3.4.3.4. Allergenicity

For the allergenicity assessment, a weight-of-evidence approach was followed, taking into account all of the information obtained on the newly expressed proteins, as no single piece of information or experimental method yields sufficient evidence to predict allergenicity (Codex Alimentarius, 2009; EFSA GMO Panel, 2011a; Regulation (EU) No 503/2013). In addition, when known functional aspects of the newly expressed protein or structural similarity to known adjuvants may indicate an adjuvant activity, the possible role of these proteins as adjuvants is considered. When newly expressed proteins with a potential adjuvant activity are expressed together, possible interactions increasing adjuvanticity and impacting the allergenicity of the GM crop are assessed.

Assessment of allergenicity of the newly expressed proteins

For allergenicity, the GMO Panel has previously evaluated the safety of the Cry1A.105, Cry2Ab2, PMI, Vip3Aa20 and CP4 EPSPS (including its variant CP4 EPSPS L214P) proteins individually, and no concerns on allergenicity were identified in the context of the applications assessed (Table 2). No new information on allergenicity of these proteins that might change the previous conclusions of the GMO Panel has become available. Based on the current knowledge, and as none of the newly expressed proteins showed allergenicity, no reasons for concerns regarding the simultaneous presence of these newly expressed proteins in this four-event stack maize affecting their allergenicity are expected.

For adjuvanticity, the Bt protein Cry1Ac have been suggested to possess adjuvant activity based on animal studies on Cry1Ac when applied at relatively high doses (e.g. Vázquez et al., 1999). The GMO Panel has previously evaluated the safety of the Cry1A.105, Cry2Ab and Vip3Aa20 proteins and no concerns on adjuvanticity were identified in the context of the applications assessed (Table 2). The levels of individual Bt proteins in this four-event stack maize are comparable to those in the respective single maize events (see Section 3.4.1.3). From the limited experimental evidence available, the GMO Panel did not find indications that the presence of the Bt proteins at the levels expressed in this four-event stack maize might act as adjuvants with the potential to enhance a specific immunoglobulin E (IgE) response and to favour the development of an allergic reaction.

Assessment of allergenicity of GM plant products

The EFSA GMO Panel regularly reviews the available publications on food allergy to maize. However, maize is not considered a common allergenic food (OECD, 2002).[22] Therefore, the EFSA 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 above and Sections 3.4.1 and 3.4.2), the GMO Panel identified no indications of a potentially increased allergenicity of food and feed derived from the four-event stack maize with respect to that derived from the non-GM comparator.

[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.

3.4.3.5. Dietary exposure assessment of new constituents

In line with Regulation (EU) No 503/2013, the applicant provided dietary exposure estimates to CP4 EPSPS (CP4 EPSPS and CP4 EPSPS L214P), Cry1A.105, Cry2Ab2, Vip3Aa20, and PMI proteins newly expressed in MON 87427 × MON 89034 × MIR162 × 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 herbicide, the current available consumption data and feed practices, the foods and feeds currently available on the market and the described processing conditions.

Table 7 describes the protein expression levels used to estimate both human and animal dietary exposure.

Table 7: Mean values (n = 20, μg/g dry weight and μg/g fresh weight) for newly expressed proteins in grains and forage from MON 87427 × MON 89034 × MIR162 × NK603 maize treated with the intended herbicide[(a)]

Protein	Tissue/developmental stage	
	Grains/R6 (μg/g dry weight and μg/g fresh weight)	Forage/R5 (μg/g dry weight)
CP4 EPSPS[(b)]	13.0/12.0	300.0
Cry1A.105	2.4/2.1	32.0
Cry2Ab2	0.69/0.6	29.0
Vip3Aa20	59.0/51.9[(c)]	100.0
PMI	1.4/1.2[(c)]	4.4

(a): Intended herbicide: glyphosate.
(b): CP4 EPSPS levels in MON 87427 × MON 89034 × MIR162 × NK603 maize are the sum of two protein variants, CP4 EPSPS (expressed in MON 87427 and NK603) and CP4 EPSPS L214P (expressed in NK603).
(c): Fresh weight values for Vip3Aa20 and PMI proteins used to estimate human dietary exposure were calculated by multiplying the dry weight values by a dry weight correction factor of 0.88 to account for approximately 12% moisture content in the grains.

Human dietary exposure

Dietary exposure was estimated across different European countries on different population groups: young population (infants, toddlers, 'other children'), adult population (adolescents, adults, elderly and very elderly) and special populations (pregnant and lactating women).

For the purpose of estimating dietary exposure, the levels of newly expressed proteins in MON 87427 × MON 89034 × MIR162 × NK603 maize grains were derived from replicated field trials (four replicates from five locations) in the 2013 US growing season. Mean values (fresh weight) are considered as the most adequate to estimate dietary exposure (see Table 7). Since no specific consumption data were available on commodities containing, consisting of or obtained from MON 87427 × MON 89034 × MIR162 × 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).[23] Maize oil was excluded from the assessment since no proteins are expected to be present in the oil.

For the acute dietary exposure estimations, the applicant assigned to the processed commodities the mean value reported for the newly expressed proteins in maize grains. This is a conservative approach as neither recipes nor the effect of processing is considered on the final concentration of newly expressed proteins. Summary statistics from the EFSA consumption database were used.[24] Acute dietary exposure was estimated using for each population group the food commodity with the highest acute consumption among consumers only (95th or 97.5th percentile depending on the number of consumers), and multiplying this value by the mean values of CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20, and PMI proteins (EFSA, 2011). Table 8 shows the highest acute dietary exposure for the different newly expressed proteins; dietary exposure estimates ranged between 2.2 μg/kg body weight (bw) per day for Cry2Ab2 in adults (18–65 years) and 466.7 μg/kg bw per day for Vip3Aa20 in toddlers (1–3 years). The most relevant food commodities in terms of contribution to the exposure were sweet corn (toddlers) and popcorn (adults).

[23] http://www.efsa.europa.eu/en/data/food-consumption-data
[24] Summary statistics from the EFSA Comprehensive European Food Consumption Database accessed in September 2015.

Table 8: Highest acute dietary exposure to CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20, and PMI proteins (μg/kg bw per day) estimated across European dietary surveys and different age classes

	Acute dietary exposure (μg/kg bw per day)				
	CP4 EPSPS[(a)]	Cry1A.105	Cry2Ab2	Vip3Aa20	PMI
Toddlers	107.9	18.9	5.6	466.7	11.7
Adults	42.1	7.4	2.2	182.2	4.3

bw: body weight; EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase.
(a): CP4 EPSPS levels in MON 87427 × MON 89034 × MIR162 × NK603 maize are the sum of two protein variants, CP4 EPSPS (expressed in MON 87427 and NK603) and CP4 EPSPS L214P (expressed in NK603).

The GMO Panel estimated chronic dietary exposure to CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20, and PMI proteins. Individual consumption data of the relevant food commodities were retrieved from the EFSA Consumption Database, using dietary surveys with at least two days consumption and covering a total of 22 European countries.[25] Different recipes and factors were considered to estimate the amount of maize in the consumed commodities before assigning CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20, and PMI proteins levels to the relevant commodities.[26] No losses in the newly expressed proteins (NEPs) during processing were considered, except for certain commodities excluded from the exposure estimations (maize oil, corn starch, corn syrup). The 95th percentile chronic exposure (highly exposed population) was derived from the distribution of the individual dietary exposure estimates within each dietary survey and age class. Table 9 shows the chronic dietary exposure to each of the newly expressed proteins across European dietary surveys; dietary exposure ranged between 0.005 μg/kg bw per day for Cry2Ab2 protein in elderly and very elderly population (> 65 years) and 233.3 μg/kg bw per day for Vip3Aa20 protein in infants (< 1 year). Main average contributors to the exposure in the dietary surveys with the highest estimates were sweet corn in infants, and cornflakes in toddlers and 'Other children'.

Table 9: Range of chronic dietary exposure estimates (95th percentiles, highly exposed population) to CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20, and PMI proteins (μg/kg bw per day) across European dietary surveys and different age classes

	N	Chronic dietary exposure (μg/kg bw per day)				
		CP4 EPSPS[(a)]	Cry1A.105	Cry2Ab2	Vip3Aa20	PMI
Infants	11	0–53.9	0.0–9.3	0.0–2.8	0.0–233.3	0.0–5.4
Toddlers	14	2.9–50.2	0.5–8.6	0.2–2.6	12.7–217.0	0.3–5.0
Other children	19	8.0–44.0	1.4–7.6	0.4–2.3	34.4–190.2	0.8–4.4
Adolescents	18	1.7–32.9	0.3–5.7	0.1–1.7	7.3–142.4	0.2–3.3
Adults	19	0.8–16.6	0.1–2.9	0.04–0.9	3.4–71.6	0.1–1.7
Elderly and very elderly	18	0.1–10.2	0.02–1.8	0.005–0.5	0.4–44.1	0.01–1.0
Special population[(b)]	4	4.9–24.0	0.9–4.1	0.3–1.2	21.1–103.8	0.5–2.4

bw: body weight; n: number of dietary surveys; EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase.
(a): CP4 EPSPS levels in MON 87427 × MON 89034 × MIR162 × NK603 maize are the sum of two protein variants, CP4 EPSPS (expressed in MON 87427 and NK603) and CP4 EPSPS L214P (expressed in NK603).
(b): Pregnant women and lactating women

Animal dietary exposure

Animal dietary exposure to CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20, and PMI proteins was estimated following the consumption of maize grain, gluten feed, gluten meal and maize forage/silage since these are the maize products entering the feed chain. A conservative scenario with 100% replacement of conventional maize products by the GM products was considered.

[25] Austria, Belgium, Bulgaria, Cyprus, the Czech Republic, Germany, Denmark, Estonia, Finland, France, the United Kingdom, Greece, Croatia, Hungary, Ireland, Italy, Latvia, the Netherlands, Portugal, Spain, Romania, and Sweden.
[26] Example: 100 g 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 47.2 μg of Vip3Aa20 per gram of maize bread as compared to 51.9 μg/g in the maize grains.

Mean levels of CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20, and PMI proteins in maize grains and forage/silage were derived from field trials conduct in the 2013 US growing season (see Table 7). To estimate the mean NEP levels in maize gluten feed and gluten meal, a factor of 2.6 and 7.1 folds respectively was applied, based on the protein content of gluten feed and gluten meal relative to maize grain (OECD, 2002), assuming that no losses of NEP occur during processing.

Dietary exposure to CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20, and PMI proteins in maize MON 87427 × MON 89034 × MIR162 × NK603 following the consumption of maize grain, gluten feed and gluten meal was provided by the applicant across different animal species (i.e. broiler, finishing pig and lactating dairy cattle), based on estimates for animal body weight, daily feed intake and inclusion rates (percentage) of maize grain, gluten feed and gluten meal in animal diets (OECD, 2009). Estimated dietary exposure was as follows:

- to CP4 EPSPS protein, 1,532 μg/kg bw per day in broiler chickens, 1,250 μg/kg bw per day in dairy cattle and 753 μg/kg bw per day in finishing pig.
- to Cry1A.105 protein, 283 μg/kg bw per day in broiler chickens, 231 μg/kg bw per day in dairy cattle and 139 μg/kg bw per day in finishing pig.
- to Cry2Ab2 protein, 81 μg/kg bw per day in broiler chickens, 66 μg/kg bw per day in dairy cattle and 40 μg/kg bw per day in finishing pig.
- to Vip3Aa20 protein, 6,955 μg/kg bw per day in broiler chickens, 5,673 μg/kg bw per day in dairy cattle and 3,416 μg/kg bw per day in finishing pig.
- to PMI protein, 165 μg/kg bw per day in broiler chickens, 135 μg/kg bw per day in dairy cattle and 81 μg/kg bw per day in finishing pig.

The GMO Panel estimated dietary exposure to CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20, and PMI proteins in maize MON 87427 × MON 89034 × MIR162 × NK603 across different livestock animal species (beef and dairy cow, lamb, breeding swine and layer) following the consumption of maize forage/silage, based on estimates for animal body weight, daily feed intake and inclusion rates of maize forage/silage in animal diets (OECD, 2009).

Estimated dietary exposure was as follows:

- to CP4 EPSPS protein, 5,760 μg/kg bw per day in beef, 6,923 μg/kg bw per day in dairy cow, 3,825 μg/kg bw per day in lamb, 1,385 μg/kg bw per day in breeding swine and 2,052 μg/kg bw per day in layer.
- to Cry1A.105 protein, 614 μg/kg bw per day in beef, 738 μg/kg bw per day in dairy cow, 408 μg/kg bw per day in lamb, 147 μg/kg bw per day in breeding swine and 219 μg/kg bw per day in layer.
- to Cry2Ab2 protein, 557 μg/kg bw per day in beef, 670 μg/kg bw per day in dairy cow, 370 μg/kg bw per day in lamb, 134 μg/kg bw per day in breeding swine and 198 μg/kg bw per day in layer.
- to Vip3Aa20 protein, 1,920 μg/kg bw per day in beef, 2,307 μg/kg bw per day in dairy cow, 1,257 μg/kg bw per day in lamb, 461 μg/kg bw per day in breeding swine and 684 μg/kg bw per day in layer.
- to PMI protein, 84 μg/kg bw per day in beef, 101 μg/kg bw per day in dairy cow, 56 μg/kg bw per day in lamb, 20 μg/kg bw per day in breeding swine and 30 μg/kg bw per day in layer.

3.4.3.6. Nutritional assessment of endogenous constituents

The intended traits of the four-event stack maize are herbicide tolerance and insect resistance, with no intention to alter nutritional parameters. Comparison of the composition of maize MON 87427 × MON 89034 × MIR162 × NK603 with the non-GM comparator and non-GM reference varieties did not identify differences that would require further safety assessment. From these data, the GMO Panel concludes that the nutritional impact of maize MON 87427 × MON 89034 × MIR162 × NK603-derived food and feed is the same as that expected from the non-GM comparator and non-GM reference varieties.

3.4.3.7. Conclusion of the food and feed safety assessment

The newly expressed proteins Cry1A.105, Cry2Ab, PMI, Vip3Aa20, CP4 EPSPS and its variant CP4 EPSPS L214P in maize MON 87427 × MON 89034 × MIR162 × NK603 do not raise safety concerns for human and animal health. Interactions between these newly expressed proteins raising food and feed safety concerns (toxicological, allergenicity and adjuvanticity) are not expected. The nutritional

impact of the four-event stack maize foods and feeds is expected to be the same as those from the non-GM comparator and non-GM reference varieties. The GMO Panel concludes that the four-event stack maize, as described in this application, is as safe as and nutritionally equivalent to the non-GM comparator and the non-GM reference varieties tested.

3.4.4. Environmental risk assessment[27]

Considering the scope of application EFSA-GMO-NL-2016-131, which excludes cultivation, the environmental risk assessment (ERA) of the four event stack maize 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 four event stack maize grains during transportation and/or processing (EFSA GMO Panel, 2010a).

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. Occasional feral GM maize plants may occur outside cultivation areas in the EU (e.g. Pascher, 2016), but 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). Field observations indicate that maize grains may survive and overwinter in some EU regions, resulting in volunteers in subsequent crops (e.g. Gruber et al., 2008; Palaudelmàs et al., 2009; Pascher, 2016). However, maize volunteers have been shown to grow weakly and flower asynchronously with the maize crop (Palaudelmàs et al., 2009). Thus, the establishment and survival of feral and volunteer maize in the EU is currently limited and transient.

It is unlikely that the intended traits of event the four-event stack maize will provide a selective advantage to maize plants, except when they are exposed to glyphosate-containing herbicides or infested by insect pests that are susceptible to the Cry1A.105, Cry2Ab2 and/or Vip3Aa20 proteins. However, this fitness advantage will not allow the GM plant to overcome other biological and abiotic factors (described above) limiting plant's persistence and invasiveness. Therefore, the presence of the intended traits will not affect the persistence and invasiveness of the GM plant.

In conclusion, the GMO Panel considers it very unlikely that the four-event stack maize will differ from conventional maize hybrid varieties in its 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 grains of the four-event stack maize.

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 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 (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 MON 87427, MON 89034, MIR162 and NK603 do not reveal any new DNA sequence that could provide sufficient length and identity which could facilitate HGT by double homologous recombination, confirming the conclusions of previous Scientific Opinions (EFSA GMO Panel, 2017a,b, 2019a,b,c).

[27] Dossier: Part II – Section 5.

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 four-event stack maize to bacteria does not raise any environmental safety concern.

Plant-to-plant gene transfer

The potential for occasional feral GM maize MON 87427 × MON 89034 × MIR162 × 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).

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-2016-131 (no cultivation), potential interactions of occasional feral four-event stack maize 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 four-event stack maize 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 or Vip proteins (as mentioned in Section 3.4.1.4) 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 four-event stack maize 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 the four-event stack maize would differ from conventional maize varieties in its ability to persist under European environmental conditions. Considering the scope of the application EFSA-GMO-NL-2016-131, interactions of occasional feral four-event stack maize 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 comparative analysis, and the routes and levels of exposure, the GMO Panel concludes that the four-event stack maize would not raise safety concerns in the event of accidental release of viable GM maize grains into the environment.

3.4.5. Conclusion on the four-event stack maize MON 87427 × MON 89034 × MIR162 × NK603

No new data on the four single maize events MON 87427, MON 89034, MIR162 and NK603 that would lead to a modification of the original conclusions on their safety were identified.

The combination of maize events MON 87427, MON 89034, MIR162 and NK603 in the four-event stack maize did not give rise to issues pertaining to the molecular, agronomic/phenotypic or compositional characteristics of the four-event stack maize that would be of concern for food and feed safety and nutrition.

The newly expressed proteins in the four-event stack maize do not raise safety concerns for human and animal health and the environment in light of the scope of this application.

No indications of interactions between the events based on the biological functions of the newly expressed proteins that would raise a safety issue were identified in maize MON 87427 × MON 89034 × MIR162 × NK603. Comparison of the levels of the newly expressed proteins between the four-event stack maize and those of the single maize events did not reveal an interaction at protein expression level.

Considering the combined traits and their interactions, the outcome of the comparative analysis, and the routes and levels of exposure, the GMO Panel concludes that maize MON 87427 × MON 89034 × MIR162 × NK603 would not raise safety concerns in the event of accidental release of viable GM maize grains into the environment.

No scientific information that could change the conclusions on this four-event stack maize was retrieved through systematic literature searches covering the 10 years before submission of the application and the period since the time of validity of the application. The GMO Panel concludes that maize MON 87427 × MON 89034 × MIR162 × NK603, as described in this application, is nutritionally equivalent to and as safe as the comparator and the non-GM reference varieties tested.

3.5. Risk assessment of the subcombinations[28]

Subcombinations previously assessed in the frame of other applications are discussed in Section 3.5.1. The strategy followed for the subcombinations that have not been previously assessed (Section 3.5.2) has been described by the GMO Panel.[29] In this case, 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).

3.5.1. Subcombinations previously assessed

The GMO Panel has previously assessed four subcombinations (3 two-events stacks and a three-event stack; see Table 2) and did not identify any safety concern. Literature searches covering the 10 years before submission of application EFSA-GMO-NL-2016-131 (February 2006-February 2016) 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. Consequently, the GMO Panel considers that its previous conclusions on these subcombinations remain valid.

3.5.2. Subcombinations not previously assessed

Of the 10 subcombinations included in the scope of application EFSA-GMO-NL-2016-131, six have not been assessed by the GMO Panel (see Table 10). No experimental data were provided for these maize stacks.

[28] Dossier: Part II – Section 7; additional information: 24/5/2018 and 23/3/2019.
[29] 115th GMO Panel meeting (Annex 1 of the minutes: http://www.efsa.europa.eu/sites/default/files/event/170517-m.pdf).

Table 10: Subcombinations not previously assessed and covered by the scope of application EFSA-GMO-NL-2016-131

Degree of stacking	Events
Three-event stack maize	MON 87427 × MON 89034 × MIR162
	MON 87427 × MIR162 × NK603
	MON 89034 × MIR162 × NK603
Two-event stack maize	MON 87427 × MIR162
	MON 89034 × MIR162
	MIR162 × NK603

3.5.2.1. Stability of the events

The genetic stability of the inserted DNA over multiple generations in the single maize events has been previously demonstrated (see Table 2). Integrity of the events was demonstrated in maize MON 87427 × MON 89034 × MIR162 × NK603 (Section 3.4.1.2) and the previously assessed maize subcombinations (EFSA GMO Panel, 2009, EFSA GMO Panel, 2017a). The GMO Panel finds no reasons to expect the loss of integrity of the events in the maize subcombinations not previously assessed (see Table 10).

3.5.2.2. Expression of the events

The GMO Panel assessed whether the combination of any 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 interaction between 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 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 except for CP4 EPSPS, which showed, in general, the expected higher level in the stack resulting from the combination of the single events MON 87427 and NK603 (Section 3.4.1.3 and Appendix A). Therefore, there was no indication of an interaction at protein expression level. In addition, expression data from the two-event stack maize MON 89034 × NK603 (EFSA GMO Panel, 2009) and the three-event stack maize MON 87427 × MON 89034 × NK603 (EFSA GMO Panel, 2017a) were similar to those observed in each of the single maize events or showed in general the expected higher levels for CP4 EPSPS resulting from the combination of the single events MON 87427 and NK603 both producing CP4 EPSPS protein in the three-event stack. This supports the conclusion that interactions affecting expression levels of the newly expressed proteins are not expected in the maize subcombinations not previously assessed and included in the scope of application EFSA-GMO-NL-2016-131.

3.5.2.3. Potential interactions between the events

The GMO Panel assessed the potential for interactions between maize events in the six subcombinations not previously assessed (Table 10), taking into consideration the 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/feed or environmental safety between these proteins in those subcombinations. The GMO Panel also took into account all the intended and potential unintended effects considered in the assessment of the four single events, the previously assessed subcombinations (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 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 subcombinations, the GMO Panel considers that its previous conclusions on these maize subcombinations remain valid. For the remaining six subcombinations included in the scope of application EFSA-GMO-NL-2016-131, no

experimental data have been provided. For these subcombinations, the GMO Panel assessed the possibility of interactions between 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 subcombinations and the four-event stack maize.

3.6. Post-market monitoring[30]

3.6.1. Post-market monitoring of GM food/feed

The GMO Panel concluded that the four-event stack maize, as described in this application, is nutritionally equivalent to and as safe as the non-GM comparator and the non-GM reference varieties tested (Section 3.4.3.7). Four of the subcombinations have been previously assessed and no safety concerns were identified. The six subcombinations not previously assessed and included in the scope of application EFSA-GMO-NL-2016-131 are expected to be as safe as and nutritionally equivalent to the single maize events, the previously assessed maize subcombinations and the four-event stack maize (Section 3.5.3). 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) 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: (1) the description of a monitoring approach involving operators (federations involved in import and processing), reporting to the applicant, via a centralised system, any observed adverse effect(s) of GMOs on human health and the environment; (2) a coordinating system established by EuropaBio for the collection of information recorded by the various operators; and (3) the review of relevant scientific publications retrieved from literature searches (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a PMEM report on an annual basis and a final report at the end of the authorisation period.

The GMO Panel considers that the scope of the PMEM plan provided by the applicant is consistent with the intended uses of 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.

In the context of annual PMEM reports, the applicant should improve future literature searches according to the GMO Panel recommendations given in Section 3.3.

3.6.3. Conclusion 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 maize MON 87427 × MON 89034 × MIR162 × NK603.

4. Overall conclusions

The GMO Panel was asked to carry out a scientific assessment of maize MON 87427 × MON 89034 × MIR162 × NK603 and subcombinations for import, processing and food and feed uses in accordance with Regulation (EC) No 1829/2003.

[30] Dossier: Part II – Sections 4 and 6; additional information: 29/9/2016 and 13/12/2016.

No new information on the four single maize events MON 87427, MON 89034, MIR162 and NK603 that would lead to a modification of the original conclusions on their safety were identified.

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, is as safe as and nutritionally equivalent to its non-GM comparator and the non-GM reference varieties tested.

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 on the four subcombinations previously assessed (3 two-event stacks and a three-event stack) that would lead to a modification of the original conclusions on their safety were identified, the GMO Panel considers that its previous conclusions on these maize stacks remain valid. For the remaining six subcombinations included in the scope of application EFSA-GMO-NL-2016-131, no information has been provided. The GMO Panel assessed possible interactions between the events in the six subcombinations, and concludes that these combinations of events MON 87427, MON 89034, MIR162 and NK603 would not raise safety concerns. These subcombinations are therefore expected to be as safe as and nutritionally equivalent to the maize single events, the previously assessed subcombinations 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 MON 87427 × MON 89034 × MIR162 × NK603 and its subcombinations. In the context of annual PMEM reports, the applicant should improve future literature searches according to the GMO Panel recommendations.

In addition, the GMO Panel considered the additional unpublished studies listed in Appendix B. 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 concerns for foods and feeds from the four-event stack maize and all its subcombinations, the GMO Panel considers that post-market monitoring of these products is not necessary. The post-market environmental monitoring 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 MON 87427 × MON 89034 × MIR162 × NK603 and its subcombinations, as described in this application, are as safe as the non-GM comparator and the tested non-GM reference varieties with respect to potential effects on human and animal health and the environment.

Documentation as provided to EFSA

- Letter from the Competent Authority of Netherlands received on 18 February 2016 concerning a request for authorisation of the placing on the market of maize MON 87427 × MON 89034 × MIR162 × NK603 (EFSA-GMO-NL-2016-131) submitted in accordance with Regulation (EC) No 1829/2003 by Monsanto Europe S.A./N.V.
- Application EFSA-GMO-NL-2016-131 validated by EFSA, 31 May 2016
- Request for supplementary information to the applicant (on behalf of EURL-GMFF), 7 June 2016
- Request for supplementary information to the applicant, 9 June 2016
- Receipt of supplementary information from the applicant, 6 July 2016
- Request for supplementary information to the applicant, 3 August 2016
- Receipt of supplementary information from the applicant, 29 September 2016
- Request for supplementary information to the applicant, 8 November 2016
- Receipt of supplementary information from the applicant, 9 January 2017
- Receipt of supplementary information from the applicant, 10 January 2017
- Request for supplementary information to the applicant, 24 February 2017
- Request for supplementary information to the applicant, 11 April 2017
- Receipt of supplementary information from the applicant, 21 April 2017
- Request for supplementary information to the applicant, 18 May 2017
- Receipt of supplementary information from the applicant, 11 July 2017
- Receipt of supplementary information from the applicant, 18 July 2017
- Request for supplementary information to the applicant, 1 August 2017

- Request for supplementary information to the applicant, 1 September 2017
- Receipt of supplementary information from the applicant, 14 September 2017
- Receipt of supplementary information from the applicant, 25 September 2017
- Receipt of spontaneous information from the applicant, 20 December 2017
- Request for supplementary information to the applicant, 15 February 2018
- Request for supplementary information to the applicant, 23 March 2018
- Receipt of supplementary information from the applicant, 24 May 2018
- Receipt of spontaneous information from the applicant, 24 September 2018
- Receipt of supplementary information from the applicant, 16 October 2018
- Request for supplementary information to the applicant, 6 November 2018
- Request for supplementary information to the applicant, 8 November 2018
- Receipt of supplementary information from the applicant, 8 November 2018
- Receipt of supplementary information from the applicant, 23 November 2018
- Receipt of supplementary information from the applicant, 30 November 2018
- Request for supplementary information to the applicant, 21 December 2018
- Request for supplementary information to the applicant, 18 January 2019
- Request for supplementary information to the applicant, 24 January 2019
- Receipt of supplementary information from the applicant, 22 March 2019
- Receipt of supplementary information from the applicant, 25 March 2019
- Receipt of supplementary information from the applicant, 26 March 2019

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Abbreviations

ADF	acid detergent fibre
bw	body weight
CaMV	cauliflower mosaic virus
CRM	comparative relative maturity
CTP	Chloroplast transit peptide
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ERA	environmental risk assessment
FMV	Figwort Mosaic Virus
GM	genetically modified
GMO	genetically modified organism
GMO Panel	EFSA Panel on Genetically Modified Organisms
HGT	horizontal gene transfer
HR	homologous recombination
IgE	immunoglobulin E
LOD	limit of detection
NDF	neutral detergent fibre
NEP	newly expressed protein
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PCR	polymerase chain reaction
PMEM	post-market environmental monitoring
PMI	phosphomannose isomerase
T-DNA	transfer-deoxyribonucleic acid
TDF	total dietary fibre
UTR	untranslated region

Appendix A – Protein expression data

Means, standard deviation and ranges of protein levels (μg/g dry weight) from maize MON 87427 × MON 89034 × MIR162 × NK603 (treated with glyphosate), MON 87427 (treated with glyphosate), MON 89034 (not treated), MIR162 (not treated) and NK603 (treated with glyphosate) from field trials performed in the US in 2013[a]

Protein	Event(s)	Leaf (V2–V4)	Whole Plant (V10–V11)	Forage(R5)	Root(V2–V4)	Root(R5)	Grain(R6)	Pollen(R1)
CP4 EPSPS[b]	**MON 87427 × MON 89034 × MIR162 × NK603**	1100[c] ± 400[d] (260–1700)[e]	430 ± 140 (280–790)	300 ± 41 (240–390)	390 ± 99 (240–610)	160 ± 18 (120–200)	13 ± 2.8 (9.4–18)	290 ± 51 (180–350)
	MON 87427	950 ± 150 (620–1300)	380 ± 110 (240–630)	160 ± 31 (98–220)	260 ± 44 (190–330)	120 ± 34 (71–180)	5.4 ± 1.7 (2.0 – 8.9)	< LOD[f],[g]
	NK603	170 ± 120 (79–480)	110 ± 19 (71–140)	81 ± 17 (49–100)	130 ± 43 (70–220)	72 ± 21 (48–130)	6.9 ± 1.1 (4.5–8.5)	290 ± 56 (190–380)
Cry1A.105	**MON 87427 × MON 89034 × MIR162 × NK603**	450 ± 91 (320–630)	94 ± 42 (49–180)	32 ± 13 (17–59)	64 ± 12 (48–96)	15 ± 3.5 (8.4–21)	2.4 ± 0.86 (1.4–3.9	11 ± 3.0 (5.9–18)
	MON 89034	520 ± 100 (380–690)	91 ± 31 (50–170)	39 ± 15 (13–68)	59 ± 12 (37–78)	14 ± 3.9 (4.0–20)	2.2 ± 0.65 (1.3–3.2)	11 ± 3.4 (5.9–16)
Cry2Ab2	**MON 87427 × MON 89034 × MIR162 × NK603**	130 ± 39 (49–210)	31 ± 13 (16–58)	29 ± 5.0 (21–43)	42 ± 13 (18–70)	11 ± 3.0 (2.9–16)	0.69 ± 0.17 (0.50–1.0)	0.57 ± 0.13 (0.35–0.81)
	MON 89034	110 ± 36 (62–190)	28 ± 8.7 (15–53)	31 ± 6.1 (20–42)	36 ± 19 (15–77)	13 ± 2.7 (10–20)	0.81 ± 0.25 (0.43–1.7)	0.60 ± 0.11 (0.40–0.77)
Vip3Aa20	**MON 87427 × MON 89034 × MIR162 × NK603**	220 ± 62 (150–360)	95 ± 30 (73–170)	100 ± 22 (68–150)	100 ± 27 (60–140)	56 ± 23 (30–110)	59 ± 14 (41–95)	91 ± 7.8 (82–110)
	MIR162	270 ± 53 (190–350)	94 ± 22 (70–150)	110 ± 14 (83–130)	110 ± 25 (65–150)	54 ± 25 (17–110)	50 ± 16 (18–76)	87 ± 11 (68–110)
PMI	**MON 87427 × MON 89034 × MIR162 × NK603**	11 ± 2.3 (7.8–15)	5.6 ± 1.5 (0.40–9.3)	4.4 ± 0.90 (3.2–6.8)	6.3 ± 1.4 (3.6–7.7)	2.5 ± 0.68 (1.5–4.0)	1.4 ± 0.24 (0.87–1.9)	3.0 ± 0.54 (2.1–4.2)
	MIR162	11 ± 1.8 (8.7–15)	6.1 ± 1.1 (3.8–8.1)	4.3 ± 0.80 (3.2–5.9)	7.1 ± 1.5 (4.7–9.3)	2.2 ± 0.79 (1.0–3.6)	1.2 ± 0.36 (0.21–1.8)	2.2 ± 0.34 (1.7–2.6)

EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase; PMI: phosphomannose isomerase.
(a): Number of sample is n = 20 except for: n = 19 for whole plant (for all newly expressed proteins), forage and root/R5 (for CP4 EPSPS); n = 16 for root/V2-V4 (for Cry1A.105, Cry2Ab2, Vip3Aa20 and PMI).
(b): EPSPS levels in the maize MON 87427 × MON 89034 × MIR162 × NK603 are a sum of two protein variants, CP4 EPSPS (expressed in MON 87427 and NK603) and CP4 EPSPS L214P (expressed in NK603)
(c): Mean.
(d): Standard deviation.
(e): Range.
(f): LOD: limit of detection.
(g): Due to specific insert design, little to no CP4 EPSPS protein is expected to be produced in pollen.

Appendix B – List of additional unpublished studies performed by or on behalf of the applicant with regard to the evaluation of the safety of maize MON 87427 × MON 89034 × MIR162 × NK603 for humans, animals and the environment

Study identification	Title
MSL0022349	Immunodetection of CP4 EPSPS in Corn Grain of MON 87427 Following Heat Treatment
MSL0024759	Immunodetection of CP4 EPSPS following heat treatment
MSL0025578	Compositional Analyses of Maize Forage and Grain from MON 87427 × MON 89034 × MIR162 × NK603 Grown in the United States in 2013
MSL0026080	Phenotypic Evaluation and Environmental Interactions of Maize MON 87427 × MON 89034 × MIR162 × NK603 in 2013 U.S. Field Trials
MSL0026199	Phenotypic Evaluation of Maize MON 87427 × MON 89034 × MIR162 × NK603 with Herbicide Treatment in 2013 U.S. Field Trials
MSL0026201	Southern Blot Analyses to Confirm the Presence of MIR162 in the Combined Trait Maize Product MON 87427 × MON 89034 × MIR162 × NK603
MSL0026228	Comparison of Lipid Transfer Protein (LTP) Expression Levels from MON 87427 × MON 89034 × MIR162 × NK603 with Conventional Control Maize
MSL0026686	Amended Report for MSL0025842: Southern Blot Analyses to Confirm the Presence of MON 87427, MON89034 and NK603 in the Combined Trait Maize Product MON 87427 × MON 89034 × MIR162 × NK603
SCR-2014-0178	Compositional Analyses of Maize Forage and Grain from MON 87427 × MON 89034 × MIR162 × NK603 Grown in the United States in 2013: Individual Site Analysis