EFSA Journal 2015;13(12):4297

SCIENTIFIC OPINION

Scientific Opinion on an application by Syngenta (EFSA-GMO-DE-2009-66) for placing on the market of herbicide tolerant and insect resistant maize Bt11 × MIR162 × MIR604 × GA21 and subcombinations independently of their origin for food and feed uses, import and processing under Regulation (EC) No 1829/2003[1]

EFSA Panel on Genetically Modified Organisms (GMO)[2,3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The EFSA GMO Panel previously assessed the four single events combined to produce a four-event stack maize Bt11 × MIR162 × MIR604 × GA21 and did not identify safety concerns. In this opinion, the EFSA GMO Panel assesses the four-event stack maize and all its subcombinations independently of their origin. No new data on the single events, leading to modification of the original conclusions on their safety, were identified. The molecular, agronomic, phenotypic and compositional data on the four-event stack maize did not give rise to safety concerns and there is no reason to expect interactions between the single events impacting on the food and feed safety of the four-event stack maize. Considering the routes of exposure and limited exposure levels, the Panel concludes that this four-event stack maize would not raise safety concerns in the event of accidental release of viable grains into the environment. The EFSA GMO Panel concludes that the four-event stack maize is as safe and as nutritious as its conventional counterpart in the context of its scope. Among the 10 subcombinations, four have been assessed previously and no safety concerns were identified. For the remaining six subcombinations, the EFSA GMO Panel followed a weight-of-evidence approach, and concluded they are expected to be as safe as the four-event stack maize. For some subcombinations that could be produced by conventional crossing through targeted breeding approaches, little or no specific data were submitted, giving rise to uncertainties due to data gaps. To reduce these uncertainties and to confirm assumptions made for the assessment of these subcombinations, the EFSA GMO Panel recommends that the applicant collate relevant information, if these subcombinations were to be created via targeted breeding approaches and commercialised in the future. In this case, this information should focus on expression levels of the newly expressed proteins.

[1] On request from the Competent Authority of Germany on an application (EFSA-GMO-DE-2009-66) submitted by Syngenta, Question No EFSA-Q-2009-00444, adopted on 29 October 2015.

[2] Panel members Andrew Nicholas Birch, Josep Casacuberta, Adinda De Schrijver, Achim Gathmann, Mikolaj Antoni Gralak, Philippe Guerche, Huw Jones, Barbara Manachini, Antoine Messéan, Hanspeter Naegeli, Elsa Ebbesen Nielsen, Fabien Nogué, Christophe Robaglia, Nils Rostoks, Jeremy Sweet, Christoph Tebbe, Francesco Visioli and Jean-Michel Wal. Correspondence: GMO@efsa.europa.eu

[3] Acknowledgement: The Panel wishes to thank the members of the Working Groups on Molecular Characterisation, Food/Feed safety and Environment on GMO applications for the preparatory work on this scientific opinion, and EFSA staff: Yi Liu, Zoltán Divéki, and Sylvie Mestdagh for the support provided to this scientific opinion.

Suggested citation: EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2015. Scientific Opinion on an application by Syngenta (EFSA-GMO-DE-2009-66) for placing on the market of herbicide tolerant and insect resistant maize Bt11 × MIR162 × MIR604 × GA21 and subcombinations independently of their origin for food and feed uses, import and processing under Regulation (EC) No 1829/2003. EFSA Journal 2015;13(12):4297, 34 pp. doi:10.2903/j.efsa.2015.4297

Available online: www.efsa.europa.eu/efsajournal

KEY WORDS

GMO, maize (*Zea mays*), Bt11, MIR162, MIR604, GA21, herbicide tolerant and insect resistant, stack

SUMMARY

Following the submission of application EFSA-GMO-DE-2009-66 under Regulation (EC) No 1829/2003[4] from Syngenta, the Panel on Genetically Modified Organisms of the European Food Safety Authority (referred to hereafter as EFSA GMO Panel) was asked to deliver a scientific opinion on the safety of herbicide tolerant and insect resistant genetically modified maize Bt11 × MIR162 × MIR604 × GA21 (referred to hereafter as 'four-event stack maize') and on all its subcombinations[5] (referred to as '*subcombinations independently of their origin*' in the Commission implementing regulation (EU) No 503/2013)[6]. The scope of application EFSA-GMO-DE-2009-66 is for food and feed uses, import and processing, but excludes cultivation within the European Union (EU).

The term 'subcombination' refers to any combination of up to three of the events present in the four-event stack maize Bt11 × MIR162 × MIR604 × GA21. Subcombinations occur as segregating progeny in the harvested grains of Bt11 × MIR162 × MIR604 × GA21 (embryo and albumen), and their safety is evaluated within the assessment of the four-event stack maize Bt11 × MIR162 × MIR604 × GA21 in Section 4 of the present opinion.

'Subcombination' also refers to any combination of up to three of the events Bt11, MIR162, MIR604 or GA21 that has 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 Bt11 × MIR162 × MIR604 × GA21. These stacks, including their segregating progeny, are risk assessed in the Section 5 of the present opinion.

In accordance with the EFSA GMO Panel guidance document applicable to this application (EFSA, 2007a), "*where all single events have been assessed, the risk assessment of stacked events should focus mainly on issues related to a) stability, b) expression of the events and c) potential interactions between the events*". For application EFSA-GMO-DE-2009-66, previous assessments of the four single events (Bt11, MIR162, MIR604 and GA21) provided a basis to evaluate the four-event stack maize and the 10 subcombinations.

The four-event stack maize Bt11 × MIR162 × MIR604 × GA21 was produced by conventional crossing to combine four single maize events. Maize containing the single events, Bt11 (expressing Cry1Ab and PAT proteins), MIR162 (expressing Vip3Aa20 and PMI proteins), MIR604 (expressing mCry3A and PMI proteins) and GA21 (expressing mEPSPS protein), were assessed previously and no concerns were identified. No safety issue was identified by updated bioinformatic analyses, nor reported by the applicant concerning the four single maize events, since the publication of the scientific opinions. Consequently, the EFSA GMO Panel considers that its previous conclusions on the safety of the single maize events remain valid (Section 3).

For the four-event stack maize, the risk assessment included the molecular characterisation of the inserted DNA and the analysis of the proteins' expression. An evaluation of the comparative analyses of compositional, agronomic and phenotypic characteristics was undertaken, and the safety of the newly expressed proteins and the whole food/feed was evaluated with respect to potential toxicity, allergenicity and nutritional characteristics. Evaluation of environmental impacts and the Post-Market Environmental Monitoring (PMEM) plan was also undertaken.

The molecular data establish that the transformation events stacked in maize Bt11 × MIR162 × MIR604 × GA21 have the same molecular properties and characteristics as the

[4] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Official Journal of the European Communities, L268, 1–23.

[5] The 10 subcombinations are three-event stacks Bt11 × MIR162 × MIR604, Bt11 × MIR162 × GA21, Bt11 × MIR604 × GA21, MIR162 × MIR604 × GA21; and two-event stacks Bt11 × MIR162, Bt11 × MIR604, Bt11 × GA21, MIR162 × MIR604, MIR162 × GA21, MIR604 × GA21.

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

single transformation events. Protein expression analyses showed that the levels of the newly expressed proteins are similar in the four-event stack and the single events, with the exception of PMI. Comparison of the levels of the newly expressed proteins between the four-event stack and the respective single events did not reveal an interaction that would affect protein expression level.

The newly expressed proteins in the four-event stack maize did not raise concerns for human and animal health. The compositional data indicate that maize Bt11 × MIR162 × MIR604 × GA21 would be expected to deliver the same nutritional characteristics as its conventional counterpart. This was confirmed by the results of an animal feeding study in chickens for fattening.

The EFSA GMO Panel considers that there is no reason to expect interactions that could impact on the food and feed safety. No safety concerns are foreseen for any subcombinations of the individual events, including those not previously assessed by EFSA.

Considering the introduced traits, the outcome of the comparative analysis, the routes of exposure and the limited exposure levels, the EFSA GMO Panel concluded that this four-event stack maize would not raise safety concerns in the event of accidental release of viable GM maize grains into the environment, irrespective of possible interactions between the individual events within this four-event stack maize.

In conclusion, the EFSA GMO Panel is of the opinion that the four-event stack maize is as safe and as nutritious as its conventional counterpart and commercial maize varieties in the context of its scope.

Concerning the 10 subcombinations, EFSA GMO Panel previously assessed four of them (i.e. Bt11 × GA21, MIR604 × GA21, Bt11 × MIR604, Bt11 × GA21 × MIR604) and did not identify safety concerns. No new scientific information regarding these subcombinations was retrieved in a literature search covering the period since the publication of the respective scientific opinions. Moreover, the additional data available on protein expression, agronomic, phenotypic and compositional characteristics of maize Bt11 × MIR604 × GA21 confirmed the result of the previous assessment. Consequently, the EFSA GMO Panel considers that its previous conclusions on these four subcombinations remain valid.

For the remaining six subcombinations, with the exception of Bt11 × MIR162 × GA21, the applicant provided no experimental data. The EFSA GMO Panel used a weight-of-evidence approach to conclude on the safety of these six subcombinations, considering information from: (i) the previous assessments of the four single maize events, (ii) the assessment of the four-event stack maize, and (iii) the four subcombinations previous assessed and the newly available data. The EFSA GMO Panel is of the opinion that the six subcombinations are expected to be as safe as the four-event stack maize.

The EFSA GMO Panel considers that post-market monitoring of food/feed derived from maize Bt11 × MIR162 × MIR604 × GA21 or 10 subcombinations is not necessary, given the absence of safety concerns identified.

The EFSA GMO Panel is of the opinion that the PMEM plans provided by the applicant are in line with the scope of the four-event stack maize and the four subcombinations previously assessed. However, the PMEM plan submitted by the applicant for the four-event stack maize does not include any provision for the six subcombinations that were not previously assessed. Therefore, the EFSA GMO Panel recommends the applicant to revise the plan accordingly.

The EFSA GMO Panel did not find indication that the subcombinations, resulting from combination of any of the single events included in the four-stack, would raise safety concerns. However, for some subcombinations (Bt11 x MIR162 x MIR604, MIR162 x MIR604 x GA21, Bt11 x MIR162, MIR162 x MIR604, MIR162 x GA21) that could be produced by conventional crossing through targeted breeding approaches, little or no specific data were submitted. For these the EFSA GMO Panel has drawn conclusions on a weight-of-evidence approach, giving rise to uncertainties due to data gaps.

In order to reduce these uncertainties and to confirm assumptions made for the assessment of these subcombinations, the EFSA GMO Panel recommends that the applicant collate relevant information, if these subcombinations were to be created via targeted breeding approaches and commercialised in the future. In this case, this information should focus on expression levels of the newly expressed proteins.

In delivering its scientific opinion, the EFSA GMO Panel considered the data available on the four-event stack maize and the subcombinations, the scientific comments submitted by the Member States and the relevant scientific publications.

TABLE OF CONTENTS

BACKGROUND

On 4 March 2009, the European Food Safety Authority (EFSA) received from the Competent Authority of Germany application EFSA-GMO-DE-2009-66, for authorisation of genetically modified (GM) maize Bt11 × MIR162 × MIR604 × GA21 submitted by Syngenta within the framework of Regulation (EC) No 1829/2003 on genetically modified food and feed for food and feed uses, import and processing (EC, 2003).

After receiving the application EFSA-GMO-DE-2009-66 and in accordance with Articles 5(2)(b) and 17(2)(b) of Regulation (EC) No 1829/2003, EFSA informed Member States and the European Commission, and made the summary of the application available to the public on the EFSA website[7]. EFSA initiated a formal review of the application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of Regulation (EC) No 1829/2003. On 19 May 2009, 12 and 24 June 2009, EFSA received additional information (requested on 27 March 2009 and 3 June 2009, respectively). On 13 July 2009, EFSA declared the application valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003.

EFSA made the valid application available to Member States and the European Commission, and consulted nominated risk assessment bodies of Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC[8] following the requirements of Articles 6(4) and 18(4) of Regulation (EC) No 1829/2003 (EC, 2001, 2003), to request their scientific opinion. Member States had three months after the date of receipt of the valid application (until 22 October 2009) to make their opinion known.

The scope defined by the applicant at the time of submission was "*all food and feed products containing, consisting or produced from Bt11 x MIR162 x MIR604 x GA21 maize including products from inbreds and hybrids obtained by conventional breeding of this stacked maize product. The application also covers the import and industrial processing of Bt11 x MIR162 x MIR604 x GA21 maize for all potential uses as any other maize.*" After clarifications (letters received on 14 June 2010, 15 September 2010, 15 March 2012, 6 June 2012, 8 July 2013 and 24 July 2013), the applicant notified EFSA that the scope of EFSA-GMO-DE-2009-66 was to "*include Bt11 x MIR 162 x MIR604 x GA21 maize and all subcombinations from Bt11 x MIR 162 x MIR604 x GA21 maize independently of their origin.*"

The EFSA GMO Panel carried out an evaluation of the scientific risk assessment of maize Bt11 × MIR162 × MIR604 × GA21 and all its subcombinations. On 21 September 2009, 5 February 2010, 17 March 2010, 21 January 2011, 6 July 2012, 7 December 2012, 5 February 2013, 5 February 2014, 13 March 2014, 9 September 2014, 16 September 2014, 24 October 2014 and 18 September 2015, the EFSA GMO Panel requested additional information from the applicants. The applicants provided the requested information on 21 December 2009, 5 October 2010, 3 June 2010, 1 February 2012, 10 October 2012, 19 March 2013, 25 March 2013, 18 February 2014, 16 June 2014, 25 September 2014, 15 October 2014, 3 July 2015 and 24 September 2015, respectively. EFSA received additional information submitted by the applicant spontaneously on 10 December 2013, 28 July 2014, 21 July 2015 and 10 August 2015.

In giving its scientific opinion to the European Commission, the Member States and the applicant, and in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003 (EC, 2003), EFSA has endeavoured to respect a time limit of six months from the acknowledgement of the valid application. As additional information was requested by the EFSA GMO Panel, the time limit of six months was extended accordingly, in line with Articles 6(1), 6(2), 18(1), and 18(2) of Regulation (EC) No 1829/2003.

[7] Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2009-00444

[8] 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, 12.03.2001, p 1-38.

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

TERMS OF REFERENCE

The EFSA GMO Panel was requested to carry out a scientific risk assessment of "*maize Bt11 x MIR162 x MIR604 x GA21 and all the possible subcombinations of the single events, independently of their origin*" for food and feed uses, import and processing in accordance with Articles 6(6) and 18(6) of Regulation (EC) No 1829/2003.

Where applicable, any conditions or restrictions which should be imposed on the placing on the market and/or specific conditions or restrictions for use and handling, including post-market monitoring requirements based on the outcome of the risk assessment and, in the case of GMOs or food/feed containing or consisting of GMOs, conditions for the protection of particular ecosystems/environment and/or geographical areas should be indicated in accordance with Articles 6(5)(e) and 18(5)(e) of Regulation (EC) No 1829/2003.

The EFSA GMO Panel was not requested to give an opinion on information required under Annex II to the Cartagena Protocol. Furthermore, the EFSA GMO Panel did not consider proposals for labelling and methods of detection (including sampling and the identification of the specific transformation event in the food/feed and/or food/feed produced from it), which are matters related to risk management.

ASSESSMENT

1. Introduction

Application EFSA-GMO-DE-2009-66 covers 11 maize stacks: the four-event stack maize Bt11 × MIR162 × MIR604 × GA21 and the 10 subcombinations independently of their origin resulting from the combination of any of the single events Bt11, MIR162, MIR604 and GA21 (Table 1). The scope of this application is for food and feed uses, import and processing, but excludes cultivation within the European Union (EU).

The term 'subcombination' refers to any combination of up to three of the events present in the four-event stack maize Bt11 × MIR162 × MIR604 × GA21. Subcombinations occur as segregating progeny in the harvested grains of Bt11 × MIR162 × MIR604 × GA21 (embryo and albumen), and their safety is part of the assessment of the four-event stack maize Bt11 × MIR162 × MIR604 × GA21 in Section 4 of the present opinion.

'Subcombination' also refers to any combination of up to three of the events Bt11, MIR162, MIR604 or GA21 that has 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 Bt11 × MIR162 × MIR604 × GA21. These stacks, including their segregating progeny, are risk assessed in the Section 5 of the present opinion.

Table 1: Eleven maize stacks covered by the scope of application EFSA-GMO-DE-2009-66

Degree of stacking	Events	Unique identifiers
Four-event stack maize	Bt11 × MIR162 × MIR604 × GA21	SYN-BTØ11-1 × SYN-IR162-4 × SYN-IR6Ø4-5 × MON-ØØØ21-9
Three-event stack maize	Bt11 × MIR162 × MIR604	SYN-BTØ11-1 × SYN-IR162-4 × SYN-IR6Ø4-5
	Bt11 × MIR162 × GA21	SYN-BTØ11-1 × SYN-IR162-4 × MON-ØØØ21-9
	Bt11 × GA21 × MIR604	SYN-BTØ11-1 × MON-ØØØ21-9 × SYN-IR6Ø4-5
	MIR162 × MIR604 × GA21	SYN-IR162-4 × SYN-IR6Ø4-5 × MON-ØØØ21-9
Two-event stack maize	Bt11 × MIR162	SYN-BTØ11-1 × SYN-IR162-4
	Bt11 × MIR604	SYN-BTØ11-1 × SYN-IR6Ø4-5
	Bt11 × GA21	SYN-BTØ11-1 × MON-ØØØ21-9
	MIR162 × MIR604	SYN-IR162-4 × SYN-IR6Ø4-5
	MIR162 × GA21	SYN-IR162-4 × MON-ØØØ21-9
	MIR604 × GA21	SYN-IR6Ø4-5 × MON-ØØØ21-9

The four-event stack maize was developed to achieve insect resistance and herbicide tolerance to glyphosate- and glufosinate ammonium-based herbicides. The insect resistance confers protection against specific lepidopteran pests (e.g. *Ostrinia nubilalis* [European corn borer] and *Sesamia nonagrioides* [Mediterranean corn borer]) and coleopteran pests (*Diabrotica* spp. [corn rootworm]).

All four single maize events Bt11, MIR162, MIR604 and GA21 and four of these maize stacks have been previously assessed (Table 2) on the basis of experimental data (see Appendix A for complete list). No concerns for human and animal health or environmental safety were identified.

Table 2: Single maize events and maize stacks already assessed by the EFSA GMO Panel

Events	Application or mandate	Reference
Bt11	C/F/96/05.10	EFSA (2005)
	EFSA-GMO-RX-Bt11	EFSA (2009a)
	EFSA-M-2012–0232[(a)]	EFSA GMO Panel (2012b)
MIR162	EFSA-GMO-DE-2010-82	EFSA GMO Panel (2012a)
MIR604	EFSA-GMO-UK-2005-11	EFSA (2009b)
GA21	EFSA-GMO-UK-2005-19	EFSA (2007b)
	EFSA-GMO-RX-GA21	
Bt11 × GA21	EFSA-GMO-UK-2007-49	EFSA GMO Panel (2009)
MIR604 × GA21	EFSA-GMO-UK-2007-48	EFSA GMO Panel (2010a)
Bt11 × MIR604	EFSA-GMO-UK-2007-50	EFSA GMO Panel (2010b)
Bt11 × GA21 × MIR604	EFSA-GMO-UK-2008-56	EFSA GMO Panel (2010c)

(a): Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2012-00713.

The European Food Safety Authority (EFSA) guidance applicable to this application establishes that "*Where all single events have been assessed, the risk assessment of stacked events should focus mainly on issues related to a) stability, b) expression of the events and c) potential interactions between the events*" (EFSA, 2007a).

2. Issues raised by Member States

Issues raised by Member States on maize Bt11 × MIR162 × MIR604 × GA21 were considered in this scientific opinion and are addressed in detail in Annex G of the EFSA overall opinion[9].

3. Updated information on single events

Since the publication of the scientific opinions on the single maize events by the EFSA GMO Panel (EFSA, 2005, 2007b, 2009a, b; EFSA GMO Panel, 2012a, b), no safety issue pertaining to the four single events has been reported by the applicant.

For events MIR604 and GA21, updated nucleotide sequence information was received[10]. In the case of event MIR604, a single nucleotide difference was identified in the non-coding region of the insert as compared with the sequence originally reported in 2005. Further analyses demonstrated that this nucleotide difference had already been present in the original material used for the risk assessment of maize MIR604. In the case of event GA21, new sequence information revealed a nucleotide change in the actin promoter of copy 6, a three-base pair deletion contiguous to one nucleotide substitution within the 3′ insert flanking region and a difference in the number of complete m*epsps* (5-enolpyruvyl-shikimate-3-phosphate synthase) cassettes present within the insert. Similarly to event MIR604, further analyses demonstrated that these differences had already been present in the original material used for the risk assessment of maize GA21. The EFSA GMO Panel has performed the risk assessment of the new sequencing information for events MIR604 and GA21 in the frame of a request received from the European Commission[11] and concluded that the original risk assessments of events MIR604 and GA21 as a single and as a part of stacked events remains valid (EFSA GMO Panel, 2015a, b).

Bioinformatic analyses on the junction regions for events Bt11, MIR162, MIR604 and GA21, using the most up-to-date nucleotide sequences and methodology specified in the 2011 guidance (EFSA GMO Panel, 2011a), confirmed that there is no indication of the interruption of a known endogenous nuclear genes by any of the inserts[12]. Updated bioinformatic analyses of the amino acid sequence of the Open Reading Frames (ORFs) spanning the junction regions revealed no significant similarities to

[9] Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2009-00444.
[10] Additional information, 21/7/2015, 24/9/2015.
[11] EFSA-Q-2015-00473.
[12] Additional information: 3/7/2015.

known toxins or allergens[13]. Similarity searches of the ORFs present within the inserts revealed no significant similarities to known toxins. Similarity searches of the ORFs present within the inserts to known allergens using the criterion of 35 % identity of the amino acid sequence in a window of 80 amino acids resulted in the following above-threshold identities.

3.1. MIR162

ORF MIR162_Insert_67 shows similarity to Ara h 1 P17 precursor; ORF MIR162_Insert_83 shows similarity to wheat high molecular weight (HMW) glutenin, wheat HMW glutenin subunit Ax2*, Chain A of *Prunus dulcis* amandin and to *P. dulcis* prunin 1 precursor; ORF MIR162_Insert_109 shows similarity to Chain A of *P. dulcis* amandin, Jug r 2.0101 and to *Juglans regia* vicilin-like protein precursor. These ORFs are downstream of the ZmUbiInt promoter, but none of them contain a start codon in frame, therefore their expression is highly unlikely. ORF MIR162_Insert_342 shows similarity to Tri a *Triticum aestivum* Tri a 31.0101 triosephosphate isomerase. This ORF has a start codon; however, it has no promoter and is located on the reverse strand of the insert and, therefore, its expression is highly unlikely.

3.2. MIR604

ORF MIR604_insert_2014_92 shows similarity to wheat glutenin, peanut Ara h 1 precursor and allergen, soybean beta-conglycinin alpha prime subunit and cattle collagen alpha-2(I) chain precursor. ORF MIR604_insert_2014_107 shows similarity to wheat glutenin, wheat HMW glutenin 1By9, 5 and 10 subunits, gamma-gliadin and its B-precursor and *Brassica juncea* Bra j 1-E allergen. These ORFs are downstream of the metallothionein-like (MTL) promoter transcribing the mCry3A coding sequence, but they are in a different reading frame and they do not contain a start codon, therefore their expression is highly unlikely. ORF MIR604_insert_2014_367 shows similarity to *Bacillus lentus* subtilisin savinase. This ORF is located on the reverse strand of the intended coding sequences, it has no promoter upstream and it has no start codon; therefore, its expression is highly unlikely.

3.3. GA21

An ORF, which is present at four locations in the insert due to internal repetitions, shows similarity to ragweed homologue of Art v 1 precursor allergen. This ORF is located on the reverse strand of the m*epsps* coding sequence and it has no promoter upstream; therefore, its expression is highly unlikely.

Searches for eight-amino-acid-long exact matches to known allergens revealed that the newly expressed phosphomannose isomerase (PMI) proteins in MIR162 and in MIR604 show similarity to α-parvalbumin allergen, and an ORF in event MIR162, which is located in an alternative frame compared with the Vip3Aa20 protein, shows similarity to the rAsp f9 allergen from *Aspergillus fumigatus.* All of these matches have already been assessed by the EFSA GMO Panel and no safety issues were identified (EFSA, 2009b; EFSA GMO Panel, 2012a).

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

4. Risk assessment of maize Bt11 × MIR162 × MIR604 × GA21

4.1. Molecular characterisation

Possible interactions between the known biological functions conferred by the individual insert and interactions that would affect protein expression level are considered.

4.1.1. Genetic elements and their biological functions

Maize Bt11, MIR162, MIR604 and GA21 are combined by conventional crossing to produce the four-event stack maize Bt11 × MIR162 × MIR604 × GA21. The structure of the inserts introduced into the four-event stack maize is described in detail in the EFSA scientific opinions and no new genetic

[13] Additional information: 3/7/2015.

modifications were involved. Genetic elements in the expression cassettes of the single events are summarised in Table 3.

Table 3: Genetic elements in the expression cassettes of events stacked in maize Bt11 × MIR162 × MIR604 × GA21

Event	Promoter	5' UTR	transit peptide	Coding region	Terminator
Bt11	35S (CaMV)	IVS6 (*Zea mays*)	No	*cry1Ab* [(a)] (*Bacillus thuringiensis*)	*nos* (*Agrobacterium tumefaciens*)
	35S (CaMV)	IVS2 (*Z. mays*)	No	*pat* [(a)] (*Streptomyces viridochromogenes*)	*nos* (*A. tumefaciens*)
MIR162	ZmUbiInt (*Z. mays*)	–	No	*vip3Aa20* [(a)] (*B. thuringiensis*)	35S (CaMV)
	ZmUbiInt (*Z. mays*)	–	No	*pmi* (*Escherichia coli*)	*nos* (*A. tumefaciens*)
MIR604	MTL (*Z. mays*)	–	No	m*cry3A* [(a)] (*B. thuringiensis*)	*nos* (*A. tumefaciens*)
	ZmUbiInt (*Z. mays*)	–	No	*pmi* (*E. coli*)	*nos* (*A. tumefaciens*)
GA21	Actin 1 (*Oryza sativa*)	Actin 1 (*O. sativa*)	OTP (*Helianthus annuus*)	m*epsps* (*Z. mays*)	*nos* (*A. tumefaciens*)

(a): Codon optimised for expression in plants.
–, when no element was specifically introduced to optimise expression; OTP, optimised transit peptide; UTR, untranslated region.

There are seven newly expressed proteins in the four-event stack maize: three insecticidal proteins and four enzymes. Biological functions and intended effects conferred by these are summarised in Table 4.

Table 4: Biological functions and intended effects related to events stacked in maize Bt11 × MIR162 × MIR604 × GA21

Event	Protein	Function in donor organism	Intended effects
Bt11	Cry1Ab	Donor organism: *B. thuringiensis* var. *kurstaki* HD-1. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal protein (*cry*) genes (Schnepf et al., 1998)	Event Bt11 expresses a truncated version of the Cry1Ab protein. Cry1Ab is a protein toxic to certain lepidopteran larvae feeding on maize
	PAT	Donor organism: *S. viridochromogenes* Tü494 phosphinothricin-acetyltransferase (PAT) enzyme acetylates L-glufosinate-ammonium and thereby confers tolerance to phosphinothricin-based herbicides (Wohlleben et al., 1988)	Expression of PAT in maize Bt11 confers tolerance to glufosinate ammonium-based herbicides
MIR162	Vip3Aa20	Donor organism: *B. 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 (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 (MIR162)	Donor organism: *E. coli*. PMI catalyses the isomerisation of mannose-6-phosphate to fructose-6-phosphate and plays a role in the metabolism of mannose (Markovitz et al., 1967)	PMI (MIR162) is used as a selectable marker in maize MIR162. Mannose normally inhibits root growth, respiration and germination. Transformed cells

Event	Protein	Function in donor organism	Intended effects
			expressing PMI are able to utilise mannose as a carbon source (Negrotto et al., 2000). PMI (MIR162) differs from PMI (MIR604) at two amino acid positions[14]
MIR604	mCry3A	Donor organism: *B. thuringiensis* subsp. *tenebrionis* (Sekar et al., 1987). *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal protein (*cry*) genes (Schnepf et al., 1998)	The N-terminal 48 amino acid residues of the native Cry3A protein were deleted. In addition, a cathepsin-G protease recognition site was introduced for enhanced efficiency towards target pests (Chen and Stacy, 2003). Cry3A is a protein toxic to certain coleopteran larvae feeding on maize
	PMI (MIR604)	Donor organism: *E. coli*. PMI catalyses the isomerisation of mannose-6-phosphate to fructose-6-phosphate and plays a role in the metabolism of mannose (Markovitz et al., 1967)	PMI (MIR604) is used as a selectable marker in maize MIR604. 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). PMI (MIR604) differs from PMI (MIR162) at two amino acid positions
GA21	mEPSPS	Donor organism: *Z. mays.* EPSPS is an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Herrmann, 1995)	The amino acid sequence of the maize EPSPS enzyme was modified to render the maize tolerant to glyphosate. Expression of mEPSPS confers tolerance to glyphosate-based herbicides (Lebrun et al., 2003)

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 four single maize events was demonstrated previously (EFSA, 2005, 2007b, 2009a, b; EFSA GMO Panel, 2012a, b). Integrity of these events was demonstrated in the four-event stack maize[15] by Southern analyses in an F_1 generation representative of the commercial seed production.

4.1.3. Information on the expression of the inserts[16]

Plants were grown at a single location (five replicate blocks) under field conditions in 2006 in USA[17]. The levels of Cry1Ab, PAT, Vip3Aa20, mCry3A, PMI and mEPSPS proteins in the four-event stack maize and the four single events were quantified by enzyme-linked immunosorbent assay (ELISA). Protein levels were determined in leaves (whorl and anthesis stages), root (whorl and anthesis stages), pollen (anthesis stage), grain (physiological maturity and senescence stages) and whole plant (anthesis, physiological maturity and senescence stages). Data on grain at physiological maturity are reported and discussed below (Table 5). Due to the high similarity between the PMI in maize MIR162and the PMI in maize MIR604, the antibodies used in ELISA recognised both proteins. Therefore, it was not possible to distinguish between the PMI expressed by event MIR162 and by MIR164 in the four-event stack. The level of PMI observed in the four-event stack maize is equivalent to the sum of the PMI levels observed in the single events. As PMI levels may have an effect on carbohydrate metabolism,

[14] Dossier: Part I-Section D2(d).
[15] Dossier: Part I—Section D5 and Appendix 2.
[16] Dossier: Part I—Section D3.
[17] Dossier: Part I—Appendix 7.

the possible effects of PMI levels in the four-event stack maize are addressed in Section 4.2.3. Cry1Ab, PAT, Vip3Aa20, mCry3A and mEPSPS protein levels in the four-event stack maize were similar to the corresponding levels in the single maize events[18] (see Table 5 for protein levels in grain).

Table 5: Means and ranges of protein levels (µg/g dry weight) in grain at physiological maturity from the single event maize Bt11, MIR162, MIR604, GA21 and the four-event stack maize

Protein	Bt11 x MIR162 x MIR604 x GA21	Bt11	MIR162	MIR604	GA21
Cry1Ab	1.57 (1.15 – 2.52)	1.78 (1.19 – 2.31)	–	–	–
PAT	< LOD	< LOD	–	–	–
Vip3Aa20	140 (89.7 – 165)	–	124 (54.2 – 166)	–	–
mCry3A	0.62 (0.40 – 0.77)	–	–	0.72 (0.19 – 1.11)	–
PMI [(a)]	5.18 (3.38 – 6.54)	–	2.48 (1.08 – 3.16)	–	–
PMI [(b)]	4.74 (1.19 – 5.94)	–	–	2.33 (1.55 – 2.99)	–
mEPSPS	5.92 (2.99 – 7.69)	–	–	–	5.34 (3.62 – 7.61)

(a): The reference standard for this ELISA was purified PMI (MIR162) protein.
(b): The reference standard for this ELISA was purified PMI (MIR604) protein.
–, not assayed; LOD, limit of detection.

4.1.4. Conclusion

The molecular data establish that the transformation events stacked in maize Bt11 × MIR162 × MIR604 × GA21 have the same molecular properties and characteristics as the single transformation events. Protein expression analyses showed that the levels of the newly expressed proteins are similar in the four-event stack and the single events, with the exception of PMI. Comparison of the levels of the newly expressed proteins between the four-event stack and each of the single events did not reveal an interaction that would affect protein expression level.

Based on known mode of action of the newly expressed proteins, interaction between the Vip and Cry proteins in susceptible insects cannot be excluded (Bergamasco et al., 2013). Potential interactions are further assessed for their safety implications to human and animals in Section 4.3, and to the environment in Section 4.4.

4.2. Comparative analyses

4.2.1. Choice of comparator and production of material for the comparative assessment

Two comparative field studies were performed, one for agronomic and phenotypic characterisation and one for compositional analysis.

For the analysis of agronomic and phenotypic characteristics, the four-event stack maize and its conventional counterpart were grown in 10 locations in the USA in 2006[19]. The conventional counterpart was maize NP2673/NP2171, which had a genetic background similar to that of the four-event stack maize as indicated by their pedigrees[20]. At each location, the two types of material were grown in different plots within replicated blocks (five blocks/location) according to a randomised complete block design. Maintenance pesticide treatment was applied to all maize materials according

[18] Dossier: Part I—Appendix 7.
[19] Brookings, SD; Gaylord, MN; Janesville, WI; Maxwell, IA; Monroeville, IN; Seward, NE; El Paso, IL; Bloomington, IL ; Sadorus, IL ; Mackinaw, IL.
[20] Dossier: Part I—Section D7 and Appendix 10.

to the need at each site. No treatments of the four-event stack maize with the intended herbicides were included in the study. This experimental design allows a direct comparison between the four-event stack maize and its conventional counterpart in the presence of maintenance herbicides.

For the compositional analysis of forage and grain derived from the four-event stack maize and the conventional counterpart (maize NP2673/NP2171) were grown in six locations in the USA in 2006[21]. At each location these materials were grown in different plots within replicated blocks (three blocks/location) according to a randomised complete block design. Maintenance pesticide treatment was applied to all maize materials according to the local requirement. All plots with the four-event stack maize were treated with glyphosate- and glufosinate-ammonium-based herbicides on top of maintenance pesticides. This experimental design does not allow the effects of the genetic modification to be distinguished from the herbicide treatments.

4.2.2. Agronomic and phenotypic analysis

Nineteen parameters related to crop physiology, morphology, development, yield and biotic stress were measured[22]. Data collected for 10 of the 19 parameters were subject to an Analysis of Variance (ANOVA) across locations. The other parameters (i.e. % barren plants, % dropped ears, % emerged plants, early emergence vigour, late season intactness, leaf colour rating, late root lodging, % stalk lodging, grey leaf spot) were not subject to a formal statistical analysis because of the nature of the endpoints.

In the across-site analysis, no difference was observed between the four-event stack maize and its conventional counterpart for 8 of the 10 agronomic and phenotypic parameters. Significant differences were observed for grain test weight (converted to standard 15.5 % moisture; 72.48 ± 0.39 kg/hl for the four-event stack maize vs. 73.75 ± 0.36 kg/hl for the conventional counterpart), and percentage grain moisture (17.6 ± 0.20 % vs. 18.30 ± 0.20 %).

These significant differences are not considered relevant for human and animal health, but are further assessed for their potential environmental impact in Section 4.4.

4.2.3. Compositional analysis

Nine compositional parameters were analysed in forage and 56 in grain. These parameters were consistent with the Organisation for Economic Co-operation and Development (OECD) recommendation (OECD, 2002). Samples of forage and grain were analysed for proximates, fibre fractions and minerals. In addition, grain were analysed for starch, fatty acids, amino acids, additional minerals, pro-vitamin A and vitamins and secondary metabolites and antinutrients[23].

The grain parameters showing significant differences are shown in Table 6. The levels of these constituents were within the ranges observed in commercial non-GM maize reference varieties (Table 6). Based on the well-known biochemical roles and the characteristics of the affected parameters, and taking into account the magnitude of the observed differences, the EFSA GMO Panel considers that further assessment for potential impacts on human and animal health is not required.

[21] Dossier: Part I—Section D7 and Appendix 13.

[22] Parameters analysed as agronomic and phenotypic traits: % barren plants, % dropped ears, % emerged plants, early emergence vigour, early growth, ear height, early root lodging, % grain moisture, plant population at harvest, heat units to 50 % silking, heat units to 50 % pollen shed, late season intactness, leaf colour rating, late root lodging, plant height, % stalk lodging, grain test weight, grain yield, grey leaf spot.

[23] Measured in forage of maize Bt11 × MIR162 × MIR604 × GA21 and the conventional counterpart in the USA in 2006: Proximates (moisture, fat, ash, protein, carbohydrates (calculated)), fibre fractions (acid-detergent fibre, ADF; neutral-detergent fibre, NDF) and minerals (calcium, phosphorus). Measured in grain of Bt11 × MIR162 × MIR604 × GA21 and the conventional counterpart in the USA in 2006: Proximates (moisture, fat, ash, protein, carbohydrates (calculated)); starch; fibre fractions (ADF, NDF, total dietary fibre (TDF)); minerals (calcium, phosphorus, potassium, sodium, iron, copper, magnesium, manganese, selenium and zinc); (pro-)vitamins (β-carotene (pro-A), thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), folic acid (B9), α-tocopherol (E)); amino acids; fatty acids; and secondary metabolites and antinutrients (furfural, phytic acid, inositol, trypsin inhibitor, raffinose, ferulic acid and *p*-coumaric acid).

Table 6: Constituents (estimated means) showing significant differences between grain parameters of maize Bt11 × MIR162 × MIR604 × GA21 and its conventional counterpart NP2673/NP2171

Component	Bt11 x MIR162 x MIR604 x GA21	Conventional counterpart	Reference ranges[(a)]
Copper [mg/kg dw]	1.41	1.22	1.17 – 16.6
Potassium [mg/kg dw]	3988	3780	3090 – 5030
Stearic acid [% of total fatty acids]	1.71	1.84	1.29 – 2.19
Oleic acid [% of total fatty acids]	26.05	27.14	19.2 – 29.4
Linoleic acid [% of total fatty acids]	55.75	54.46	51.3 – 62.7
Eicosenoic acid [% of total fatty acids]	0.230	0.241	0.214 – 0.353
NDF [% dw]	9.70	9.07	4.28 – 13.9
Pyridoxine [mg/kg dw]	0.0623	0.0728	0.0439 – 0.0981
Thiamine [mg/kg dw]	0.0433	0.0416	0.0226 – 0.0520

(a): The range indicated is based on a field trial using eight hybrids of commercially available non-GM maize lines grown at six locations in the USA in 2009.
dw, dry weight.

No significant differences in the composition of forage were observed.

4.2.4. Conclusion

The EFSA GMO Panel concluded that the two differences identified (grain test weight and grain moisture) in the agronomic and phenotypic characteristics of maize Bt11 × MIR162 × MIR604 × GA21 under the tested conditions (treatment with maintenance pesticides only) and its conventional counterpart would not require further assessment regarding food and feed safety, but are further assessed for their potential environmental impact in Section 4.4.

The EFSA GMO Panel concluded that none of differences identified in the composition of grain and forage obtained from maize Bt11 × MIR162 × MIR604 × GA21 necessitated further assessment regarding food and feed safety.

4.3. Food and feed safety assessment

4.3.1. Effect of processing[24]

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 commercial non-GM maize varieties.

4.3.2. Toxicology

4.3.2.1. Toxicological assessment of newly expressed proteins[25]

Seven proteins are newly expressed in various tissues of the four-event stack maize (Section 4.1.3). The EFSA GMO Panel has previously assessed these proteins individually in the context of the single events, and no safety concern was identified.

[24] Dossier: Part I—Section D7.6.
[25] Additional information: 19/3/2013.

The expression levels of the newly expressed proteins in the four-event stack maize were similar to those of the single events with the exception of PMI. Total PMI levels were consistently higher in the tissues of the four-event stack maize than individual events. This could be expected, given the introduction of two copies of *pmi* gene. No PMI protein is detectable in conventional counterparts (below LOD or limit of quantification (LOQ)). Therefore, the fact that introduction of PMI activity in these single events did not result in changes of relevant endogenous compounds (i.e. sugars, sugar alcohols and sugar phosphates) compared with the conventional counterparts (EFSA, 2009b; EFSA GMO Panel, 2012a), indicates that these enzymes would also have no impact on carbohydrate metabolism in the four-event stack maize.

The four enzymatic proteins (PAT, mEPSPS and two PMI proteins) act on unrelated substrates, the mEPSPS protein is targeted to a specific cellular compartment (plastids). The three insecticidal proteins (Cry1Ab, mCry3A and Vip3Aa20) act through cellular receptors found in target insect species (Lee et al., 2003, 2006). It is reported that the gastrointestinal tract of mammals, including humans, lacks specific high affinity Cry protein receptors (Noteborn et al., 1995; Kuiper, 2001; Hammond et al., 2013). None of the seven proteins possessed structural similarities with known toxins to animals and humans, or showed adverse effects in the available toxicological studies. On the basis of the biological properties of the individual newly expressed proteins, there is currently no expectation for possible interactions relevant to the food and feed safety assessment of the four-event stack maize Bt11 × MIR162 × MIR604 × GA21.

4.3.2.2. Toxicological assessment of components other than newly expressed proteins

The four-event stack maize did not show any compositional difference to its conventional counterpart that would require further assessment (Section 4.2.4). No further food and feed safety assessment of components other than newly expressed proteins is required.

4.3.3. Animal studies with the food/feed derived from GM plants

A 49-day feeding study using chickens for fattening (both sexes) was provided[26]. In this study, 540 broilers (Ross, day-old) were randomly allocated into three diet treatment groups with 180 chicks per treatment (15 birds per sex per pen and 12 pens per treatment). The four-event stack maize was compared with its conventional counterpart and to a non-GM commercial variety (NC2007 maize). Grain receiving the same local agricultural management was harvested from the 2006 field trial (see Section 4.2.1 for details on field trial design). Before mixing the feed, the maize samples were analysed for proximates, amino acids and mycotoxins. The chickens were fed starter, grower and finisher diets containing 47–51 %, 54–58 % and 60–63 % of maize grain, respectively. The diets were adjusted according to the standards of the Dutch Central Feed Bureau (CVB, 2001, 2002) and the National Research Council (NRC, 1994). The concentrations of the newly expressed proteins were determined in the grain and diets by ELISA[27]. Feed and water were provided for *ad libitum* intake.

Chickens were observed twice daily for clinical signs; any death was recorded. Body weight and feed intake were measured on day 1, 16, 35 and 49. At day-50 two birds per pen were taken for carcass evaluation (dressing percentage weight of thighs, breast, wings, drums and abdominal fat). A two-way ANOVA (diet and gender) was applied, using the pen as the experimental unit. Overall mortality was low (< 3 %) with no significant difference between the groups. No significant treatment × gender interaction was detected. Final body weight (average *ca* 3.06 kg), feed:gain ratio (average 1.74) and carcass characteristics did not show significant differences between groups.

The EFSA GMO Panel concluded that this study did not detect unintended effects, and showed that the four-event stack maize is as nutritious as its conventional counterpart and the non-GM commercial variety.

[26] Dossier: Part I—Appendix 30; Additional information: 10/12/2013 (spontaneous submission).

[27] Protein concentration (µg/kg of dry weight) measured in the maize grain, starter diet, growth diet and finisher diet are Cry1Ab: 1.55, 0.47, 0.59 and 0.64, respectively; PAT always below LOD, Vip3Aa20: 87.76, 6.71, 6.84 and 19.68, respectively; Cry3A 0.25, below LOD, below LOQ and below LOQ, respectively; PMI: 3.89, 0.64, 0.74 and 1.68, respectively; mEPSPS: 7.53, 0.96, 1.11 and 2.23, respectively.

4.3.4. Allergenicity

For allergenicity assessment, a weight-of-evidence approach is followed, taking into account all of the information obtained on the newly expressed proteins, since no single piece of information or experimental method yields evidence to predict allergenicity (EFSA, 2006; Codex Alimentarius, 2009; EFSA GMO Panel, 2011a). 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 (EFSA GMO Panel, 2011a). 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.

4.3.4.1. Assessment of allergenicity of the newly expressed proteins

For allergenicity, the EFSA GMO Panel has previously evaluated the safety of the Cry1Ab, mCry3A, Vip3Aa20, PAT, EPSPS and PMI proteins individually, and no concerns on allergenicity were identified in the context of the applications assessed (see EFSA scientific opinions listed in Table 2). No new information on allergenicity of the single events that might change the previous conclusions of the EFSA GMO Panel has become available. Based on current knowledge and since none of the newly expressed proteins showed allergenicity, no concerns regarding the mixture of these newly expressed proteins in the four-event stack maize affecting allergenicity are expected.

For adjuvanticity, possible interactions between the newly expressed proteins increasing adjuvanticity and thereby potentially impacting on the allergenicity of a GM crop were considered. Bt proteins have been suggested to possess adjuvant activity, based on animal studies on Cry1Ac (e.g. Vázquez-Padrón et al., 1999; Moreno-Fierros et al., 2003; Rojas-Hernandez et al., 2004). However, at present, there is no evidence for Bt protein adjuvanticity of safety concern from the GM plants assessed so far by the EFSA GMO Panel (EFSA, 2009c; EFSA scientific opinions listed in Table 2). The levels of Bt proteins in this four-event stack maize are similar to those in the single maize events (Table 5). In addition, there is no information available on the structure or function of the individual newly expressed proteins that would suggest an adverse adjuvant effect of their mixture in the four-event stack maize, having also considered the lack of indications of adverse adjuvanticity of each individual protein in the single maize events. From the limited experimental evidence available, the EFSA GMO Panel did not find indications that the mixture of the Bt proteins in this four-event stack maize might act as adjuvants with the potential to enhance a specific IgE response and to favour the development of an allergic reaction.

4.3.4.2. Assessment of allergenicity of the whole GM plant

The EFSA GMO Panel regularly reviews the available publications on food allergy to maize (e.g. EFSA GMO Panel, 2013). However, to date, maize has not been considered to be a common allergenic food[28] (OECD, 2002). Therefore, the EFSA GMO Panel did not request experimental data to analyse the allergen repertoire of GM maize.

In the context of the present application and considering the data from the molecular characterisation, the compositional analysis and the assessment of the newly expressed proteins (see Sections 4.1 and 4.2), the EFSA GMO Panel identified no indications of safety concern regarding the overall allergenicity of the four-event stack maize.

4.3.5. Nutritional assessment of GM food/feed

The intended trait of maize Bt11 × MIR162 × MIR604 × GA21 is herbicide tolerance and insecticide resistance, with no intention to alter the nutritional parameters. Comparison of the composition of maize Bt11 × MIR162 × MIR604 × GA21 with its conventional counterpart did not identify differences that would require a safety assessment (Section 4.2.4). From these data, the nutritional characteristics of maize Bt11 × MIR162 × MIR604 × GA21-derived food and feed are not expected to

[28] Directive 2007/68/EC of the European Parliament and of the Council of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients. OJ L 310, 27.11.2007, p. 11–14.

differ from those of conventional maize varieties. This was confirmed by a feeding study in chickens for fattening (Section 4.3.3).

4.3.6. Conclusion

The newly expressed proteins in the four-event stack maize do not raise safety concerns for human and animal health, since no adverse effects in the available studies were observed, no structural similarities to known toxins were detected, and no interactions are predicted at functional level based on the known mode of action. In addition, rapid degradation of these proteins shown in *in vitro* digestibility tests suggested negligible exposure to mammalian digestive tracks by these newly expressed proteins. Similarly, the EFSA GMO Panel did not identify safety concerns regarding allergenicity or adjuvanticity with the mixture of newly expressed proteins in this four-event stack maize, or regarding the overall allergenicity of the four-event stack maize. The four-event stack maize is as nutritious as its conventional counterpart and a non-GM commercial variety.

4.4. Environmental risk assessment

The approach followed by the GMO Panel to assess possible interactions between individual events in the four-event stack maize is to consider the scope of the four-event stack maize, the modes of action of the introduced traits and the outcome of the molecular characterisation, as well as the comparative analysis.

Considering the scope (which excludes cultivation) of the four-event stack maize, the environmental risk assessment (ERA) is concerned mainly with (i) exposure of bacteria to recombinant DNA in the gastrointestinal tract of animal fed GM material and bacteria present in environments exposed to faecal material, and (ii) accidental release into the environment of viable grains of the four-event stack maize during transportation and processing.

4.4.1. Potential unintended effects on plant fitness due to the genetic modification[29]

Maize is highly domesticated, not winter hardy in many regions of Europe and generally unable to survive in the environment without appropriate management. The survival of maize plants outside cultivation areas is limited mainly by a combination of low competitiveness, absence of a dormancy phase and susceptibility to plant pathogens, herbivores and cold climate conditions. In fields, maize volunteers may arise under some environmental conditions (mild winters). Observations done in the field during harvesting indicate that grain may survive and overwinter in some regions, resulting in volunteers in subsequent crops. The occurrence of maize volunteers has been reported in Spain and other European regions (e.g. Gruber et al., 2008). However, maize volunteers have been shown to grow weakly and flower asynchronously with the maize crop (Palaudelmàs et al., 2009).

As mentioned in Section 4.2.1, a field trial was carried out in the USA in 2006 to assess the agronomic and phenotypic performance of the four-event stack maize in comparison with its conventional counterpart[30]. Significantly lower values were observed for two characteristics of the four-event stack maize, i.e. grain test weight and percentage grain moisture (Section 4.2.2). As no statistically significant differences were observed for those agronomic and phenotypic characteristics, which may affect fitness characteristics of the four-event stack maize, the EFSA GMO Panel concludes that these differences do not indicate a change in fitness of the four-event stack maize that would raise any relevant environmental safety concern.

In addition to the data presented by the applicant, the EFSA GMO Panel is not aware of any scientific report of increased spread and establishment of the four-event stack maize or maize with comparable properties or of any change in survival capacity, including overwintering[31].

[29] Dossier: Part I—Section D7.4, D9.1 and Appendix 34.
[30] Dossier: Part I—Section D7.1, D7.4 and Appendix 10; Additional information : 25/03/2013.
[31] Dossier: Part I—Section D6 and Appendix 10.

The EFSA GMO Panel considers that the inserted traits did not change the general characteristics of maize in the four-event stack maize.

Considering the scope of the four-event stack maize, the introduced traits, the outcome of the molecular characterisation, as well as the comparative analysis, and the poor ability of maize to survive outside cultivated land, the EFSA GMO Panel concludes that the four-event stack maize does not indicate an increased fitness potential compared with its conventional counterpart, if there was accidental release of viable GM maize grains into the environment.

4.4.2. Potential for gene transfer[32]

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either horizontal gene transfer of DNA or vertical gene flow via grain dispersal and cross-pollination.

4.4.3. Plant-to-bacteria gene transfer

The potential for horizontal gene transfer of the recombinant DNA of the four single events and of the four already evaluated stacks to bacteria was assessed in previous opinions (see EFSA scientific opinions listed in Table 2). No concern as a result of an unlikely, but theoretically possible, horizontal gene transfer of the recombinant genes to bacteria in the gut or other receiving environments was identified. Synergistic effects of the recombinant genes in increasing the likelihood for horizontal gene transfer, for instance combinations of recombinogenic sequences, were not identified. Therefore, the EFSA GMO Panel concludes that, in the context of its scope, the unlikely, but theoretically possible, horizontal transfer of recombinant genes from this four-event stack maize to bacteria do not raise any environmental safety concern.

4.4.4. Plant-to-plant gene transfer

Considering the scope of the four-event stack maize and the biology of maize, a possible pathway to harm pertains to the potential of occasional feral GM maize plants originating from accidental spillage of imported grains to cause adverse environmental effects through the acquisition of recombinant DNA by sexually cross-compatible plants. As pointed out above (Section 4.4.1), occurrence of feral GM maize is expected to be limited.

The extent of cross-pollination to other maize varieties will mainly depend on the scale of accidental release during transportation and processing and on successful establishment and subsequent flowering of the GM maize plant. For maize, any vertical gene transfer is limited to other *Z. mays* plants as populations of sexually compatible wild relatives of maize are not known in Europe (Eastham and Sweet, 2002; OECD, 2003).

The flowering of occasional feral GM maize plants originating from accidental release during transportation and processing is unlikely to disperse significant amounts of GM maize pollen to other maize plants. Field observations performed on maize volunteers after GM maize cultivation in Spain revealed that maize volunteers had a low vigour, rarely had cobs and produced pollen that cross-pollinated neighbouring plants at only low levels (Palaudelmàs et al., 2009). Thus, the likelihood of cross-pollination between cultivated maize and the occasional feral maize plants resulting from grain spillage is considered to be extremely low.

In conclusion, considering the scope of the four-event stack maize, the mode of action of the introduced traits, the outcome of the molecular characterisation, as well as the comparative analysis, and the poor ability of maize to survive outside cultivated land, the EFSA GMO Panel is of the opinion that the likelihood of unintended environmental effects as a consequence of spread of genes from this GM maize in Europe will not differ from that of conventional maize varieties, even in the case of treatment with the intended herbicides.

[32] Dossier: Part I—Section D6 and Appendix 34.

4.4.5. Interactions of the GM plant and target organisms[33]

Interaction between the Vip and Cry proteins in susceptible insects cannot be excluded (Bergamasco et al., 2013). However, considering the scope (which excludes cultivation) of the four-event stack maize, and the low level of exposure to the environment, potential interactions of the GM maize with target organisms were not considered a relevant issue by the EFSA GMO Panel.

4.4.6. Interactions of the GM plant with non-target organisms[34]

Considering the scope (which excludes cultivation) of the four-event stack maize, and the low level of exposure to the environment, potential interactions of GM maize plants arising from spillage of imported grains with non-target organisms were not considered a relevant issue by the EFSA GMO Panel. The EFSA GMO Panel evaluated whether the Cry1Ab, Vip3Aa20 and mCry3A proteins might potentially affect non-target organisms by entering the environment through faecal material of animals fed the four-event stack maize. Cry proteins are degraded by enzymatic activity in the gastrointestinal tract, meaning that only a very low amount of these proteins would remain intact to pass out in faeces. This was demonstrated for Cry1Ab (Einspanier et al., 2004; Lutz et al., 2005, 2006; Wiedemann et al., 2006; Guertler et al., 2008; Paul et al., 2010). Further degradation of the protein in the manure and faeces would take place because of microbiological proteolytic activity. In addition, there will be further degradation of Cry proteins in soil reducing the possibility for exposure of potentially sensitive non-target organisms. Data on degradation of Vip proteins are more limited. While proteins, including insecticidal Bt-proteins, may bind to clay minerals and humic substances in soil, thereby reducing their availability to microorganisms for degradation, there are no indications of persistence and accumulation of these proteins from GM crops in soil (reviewed by Icoz and Stotzky, 2008). The EFSA GMO Panel is not aware of evidence of released Bt-proteins causing significant negative effects on soil microorganisms.

Considering the scope of the four-event stack maize, it can be concluded that the exposure of potentially sensitive non-target organisms to the mCry3A, Cry1Ab and Vip3Aa20 proteins expressed in the four-event stack maize is likely to be very low and of no biological relevance.

4.4.7. Interactions with the abiotic environment and on biogeochemical cycles

Considering the scope of the four-event stack maize (which exclude cultivation), and the low level of exposure to the environment, potential interactions with the abiotic environment and biogeochemical cycles were not considered a relevant issue by the EFSA GMO Panel.

4.4.8. Conclusion

The EFSA GMO Panel concludes that the four-event stack maize does not indicate an increased fitness potential compared with its conventional counterpart, if there was accidental release of viable GM maize grains into the environment. Considering the scope of the GM maize, interactions with the biotic and abiotic environment were not considered to be a relevant issue. Risks associated with an unlikely but theoretically possible horizontal gene transfer of recombinant DNA from the four-event stack maize to bacteria have not been identified.

Therefore, considering the introduced traits and the outcome of the comparative analysis, the routes of exposure and the limited exposure levels, the EFSA GMO Panel concluded that this four-event stack maize would not raise safety concerns in the event of accidental release of viable GM maize grains into the environment, irrespective of possible interactions between the individual events within this four-event stack maize.

[33] Dossier: Part I—Section D9.4 and Additional information: 25/03/2013.
[34] Dossier: Part I—Section D9.5.

4.5. Conclusion on maize Bt11 × MIR162 × MIR604 × GA21

The combination of maize events Bt11, MIR162, MIR604 and GA21 in the four-event stack maize does not raise issues relating to molecular, agronomic, phenotypic or compositional characteristics that would require further assessment.

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. Comparison of the levels of the newly expressed proteins between the four-event stack and each of the single events did not reveal an interaction that manifests at protein expression level.

5. Risk assessment of the subcombinations

The risk assessment of the 10 subcombinations (Table 1) takes as its starting point the results of the assessment of the single events, the data generated for the four-event stack maize and all the additional data available for the subcombinations.

The EFSA GMO Panel assessed to what extent a combination of any of these events resulting in stacks with fewer than four events (see Table 1) could result in interactions manifesting at protein or trait expression level that were not observed in the four-event stack (e.g. because of masking). The potential for such interactions was addressed by investigating the known biological functions of the newly expressed proteins, and new data submitted.

5.1. Subcombinations previously assessed

There are four stacks that have been assessed previously by the EFSA GMO Panel: one three-event stack (maize Bt11 × MIR604 × GA21) and three two-event stacks (maize Bt11 × GA21, maize MIR604 × GA21 and maize Bt11 × MIR604). No safety concerns were identified (EFSA GMO Panel, 2009, 2010a, b, c). For the three-event stack Bt11 × MIR604 × GA21, the applicant provided additional data[35].

5.1.1. Subcombinations with no new data

No new scientific information regarding the three two-event stacks was retrieved in a literature search covering the period since the publication of the scientific opinions [36]. Consequently, the EFSA GMO Panel considers that its previous conclusions on these stacks remain valid (EFSA GMO Panel, 2009, 2010a, b).

5.1.2. Subcombination with new data

The EFSA GMO Panel assessed the additional information pertaining to the triple-event stack maize Bt11 × MIR604 × GA21[37,38].

5.1.2.1. Expression of the inserts

Protein expression data supporting the previous EFSA assessment derive from field trials carried out in the USA in 2006 indicated that the levels of proteins in the stack are similar to levels in plants containing the single maize events.

Additional protein expression data have been supplied for maize Bt11 × MIR604 × GA21 derived from field trials carried out in Romania and Spain in 2008, in which no glyphosate- or glufosinate-

[35] Additional information: 18/2/2014.
[36] Additional information: 10/10/2012 and 14/10/2014.
[37] Additional information: 18/2/2014.
[38] Additional information: 18/2/2014.

ammonium-based herbicide treatment was applied[39]. Data were provided for leaves (whorl, anthesis and maturity stages), roots (whorl, anthesis and maturity stages), pollen (anthesis stage) and grain (maturity stage). In these studies, the levels of the Cry1Ab, PAT, mCry3A, PMI and mEPSPS in the three-event stack were compared with the corresponding levels in the single maize events. This comparison did not reveal an interaction that would affect protein expression level in a way that it would require further assessment.

5.1.2.2. Comparative analysis

Additional information from field trials for agronomic, phenotypic and compositional data were obtained for the three-event stack maize and its conventional counterpart grown in six locations in the EU (three in Spain and three in Romania) in 2008[40]. In addition, agronomic and phenotypic data were collected in 2009 at seven locations (two in the Czech Republic, two in Spain and three in Romania). For both trials, maize Bt11 × MIR604 × GA21 was treated with glyphosate- and glufosinate-ammonium-based herbicides on top of the maintenance pesticides.

No differences in agronomic and phenotypic data of maize Bt11 × MIR604 × GA21 and its conventional counterpart, requiring further assessment were identified, other than the significant differences observed for plant height. These differences are further assessed for their potential environmental impact in Section 5.2.1.3.

Significant differences in grain composition were identified for 14 parameters; increased levels of ADF, TDF, thiamine, riboflavin, α-tocopherol, arachidic acid and inositol; decreased levels of zinc; palmitic, stearic, oleic, linoleic, linolenic and eicosenoic fatty acids. The mean levels fell within the ranges of conventional maize published in the literature or reported by OECD (2002). Therefore, none of the differences observed in the composition requires further assessment with regard to safety.

No significant differences were identified in the composition of forage.

5.1.2.3. Environmental risk assessment

The across-site analysis of the 2009 field trials showed statistically significant difference in plant height. The observed differences were not consistent across sites. Moreover, in the across-site analysis, the observed differences showed a lower plant height of the triple-event stack maize compared with its conventional counterpart.

Considering the scope of the triple-event stack maize, and available evidence and the poor ability of maize to survive outside cultivated land, the EFSA GMO Panel concludes that the triple-event stack maize does not indicate an increased fitness potential compared with its conventional counterpart. Considering the scope of the three-event stack maize, possible interactions between the events that may impact on the environment are not considered to be a safety issue.

5.1.3. Conclusion on the subcombinations previously assessed

No new scientific information regarding these four stacks was retrieved in a literature search covering the period since the publication of the scientific opinions. Moreover, the additional data available on protein expression, agronomic, phenotypic and compositional characteristics of maize Bt11 × MIR604 × GA21 confirmed the result of the previous assessment. Consequently, the EFSA GMO Panel considers that its previous conclusions on these stacks remain valid.

5.2. Subcombinations not previously assessed

There are six subcombinations that were not previously assessed by the EFSA GMO Panel. Data were provided for one three-event stack maize Bt11 × MIR162 × GA21, but not for the others (maize Bt11 × MIR162 × MIR604, MIR162 × MIR604 × GA21, Bt11 × MIR162, MIR162 × MIR604, MIR162 × GA21).

[39] Additional information: 18/2/2014 (Appendices 8.1 and 8.2).
[40] Additional information: 18/2/2014 (Appendix 14.1).

5.2.1. Subcombination with data

The EFSA GMO Panel assessed the additional information pertaining to the three-event stack maize Bt11 × MIR162 × GA21[41].

5.2.1.1. Expression of the inserts

The levels of Cry1Ab, PAT, Vip3Aa20, PMI (MIR162) and mEPSPS proteins in maize Bt11 × MIR162 × GA21 were compared with the corresponding levels in single maize events[42]. Plants were grown at a single location (five replicated blocks) under field conditions in 2006 in USA. Protein levels were determined in leaves (whorl, anthesis and physiological maturity stages), root (whorl, anthesis and physiological maturity stages), pollen (anthesis stage), grain (physiological maturity stage) and whole plant (anthesis, physiological maturity and senescence stages). Data on grain at physiological maturity are reported in Table 7. Comparison of the levels of the newly expressed proteins did not reveal an interaction that would affect protein expression level in a way that it would require further assessment.

Table 7: Means and ranges of protein levels (µg/g dry weight) in grain at physiological maturity from maize Bt11, MIR162, GA21 and the three-event stack maize

Event/protein	Bt11 × MIR162 × GA21	Bt11	MIR162	GA21
Cry1Ab	6.79 (4.85–10.6)	6.91 (4.35–10.7)	–	–
PAT	< LOD	< LOD	–	–
Vip3Aa20	83.8 (59.2–102)	–	83.8 (56.4–108)	–
PMI (MIR162)	1.77 (1.21–2.61)	–	1.84 (1.11–2.58)	–
mEPSPS	6.76 (3.53–8.57)	–	–	6.57 (5.35–8.76)

–, Not assayed; LOD, limit of detection.

5.2.1.2. Comparative analysis

The applicant provided compositional data for maize Bt11 × MIR162 × GA21 and its conventional counterpart from the same field trials in the USA in 2006[43]. Significant differences across sites were observed for increased levels of carbohydrates and decreased levels of phosphorus in forage. In grain, significant differences were observed for increased levels of copper, beta-carotene, thiamine and nicotinamide, and decreased levels of pyridoxine in maize Bt11 × MIR162 × GA21; increased levels of all amino acids, except for methionine, lysine, tryptophan and cysteine, as well as decreased levels of stearic acid, oleic acid, and increased levels of phytic acid. Their mean levels fell within the ranges reported for maize in literature (e.g. OECD, 2002; Reynolds et al., 2005). Therefore, none of the differences observed in the composition requires further assessment with regard to safety.

No data on agronomic and phenotypic characteristics on this three-event stack maize were provided.

5.2.2. Subcombinations with no data

Integrity of the inserts was demonstrated in the four-event stack (Bt11 × MIR162 × MIR604 × GA21, Section 4.1.2). This was confirmed by results from three two-event stacks (Bt11 × GA21, Bt11 × MIR604, MIR604 × GA21) and from two three-event stacks (Bt11 × MIR162 × GA21[44] and

[41] Additional information: 18/2/2014.
[42] Dossier: Part I—Appendix 8.
[43] Dossier: Part I—Appendix 14.
[44] Dossier: Part I—Section D5 and Appendix 3.

Bt11 × MIR604 × GA21). Therefore, the EFSA GMO Panel finds no reasons to expect the loss of integrity in any of the subcombinations.

The levels of Cry1Ab, PAT, mCry3A, Vip3Aa20 and mEPSPS proteins in the grain from the four-event stack (Bt11 × MIR162 × MIR604 × GA21, Section 4.1.3) fell in the ranges observed for the single maize events, with the exception of PMI, which is higher in the four-event stack where both MIR604 and MIR162 events are present. These results did not reveal an interaction that would affect protein expression level in a way that it would require further assessment. This was confirmed by results from three two-event stacks (Bt11 × GA21, Bt11 × MIR604, MIR604 × GA21) and from two three-event stacks (Bt11 × MIR162 × GA21[45] and Bt11 × MIR604 × GA21[46]). The EFSA GMO Panel finds no reasons to expect a different outcome for any of the subcombinations.

5.2.3. Conclusion on subcombinations not previously assessed

No indication of interactions between the events based on biological functions of the newly expressed proteins that would raise a safety issue was identified in the four-event stack maize. In particular, there is no biological basis to suggest that the presence of one protein may mask or enhance the effects of the others. Consequently, there is no reason to expect such interactions between these proteins in the 10 subcombinations involving fewer than four events. This conclusion is supported by data on genetic integrity and protein expression from the five stacks for which such data were available.

It is not expected that any combination of the newly expressed proteins would impact on the gross composition and consequently the nutritional characteristics of the maize variety into which they are introduced. This was shown by the comparative analyses of the four-event stack maize and confirmed by the comparative analyses of five stacks with their conventional counterparts.

Considering the scope of the application, the mode of action of the introduced traits, the data available for various stacks and the poor ability of maize to survive outside cultivated land, the EFSA GMO Panel is of the opinion that different combinations of these events would not raise environmental concerns.

These six subcombinations are expected to be as safe as the four-event stack maize.

6. Post-market monitoring

6.1. Post-market monitoring of GM food/feed

The EFSA GMO Panel considers that post-market monitoring of food/feed derived from maize Bt11 × MIR162 × MIR604 × GA21 or 10 subcombinations is not necessary, given the absence of safety concerns identified.

6.2. Post-market environmental monitoring

The objectives of a PMEM plan, according to Annex VII of Directive 2001/18/EC, are: (1) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in the ERA are correct and (2) to identify the occurrence of adverse effects of the GMO, or its use, on human health or the environment which were not anticipated in the ERA.

Monitoring is also related to risk management, and thus a final adoption of the PMEM plan falls outside the mandate of the EFSA GMO Panel. However, the EFSA GMO Panel gives its opinion on the scientific quality 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 (Section 4.4.6) and four of its stacks (EFSA GMO Panel, 2009, 2010a, b, c), no case-specific monitoring is required.

[45] Dossier: Part I—Appendix 8.
[46] Additional information: 18/2/2014 (Appendices 8.1 and 8.2).

The PMEM plans proposed by the applicant for the four-event stack maize[47] or the four already assessed stacks[48] (EFSA GMO Panel, 2009, 2010a, b, c) include: (1) the description of a monitoring approach involving operators (federations involved in maize import and processing), reporting to applicants, 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 the information recorded by the various operators and (3) the use of networks of existing surveillance systems (Lecoq et al., 2007; Windels et al., 2008). The applicant proposes to submit a PMEM report on an annual basis.

The EFSA GMO Panel is of the opinion that the scope of the PMEM plans provided by the applicant is in line with the scope of the four-event stack maize and the four already assessed stacks. The EFSA GMO Panel agrees with the reporting intervals proposed by the applicant in its PMEM plans. However, the PMEM plan submitted by the applicant for the four-event stack maize does not include any provision for the six stacks assessed in this opinion (Section 5.2). Therefore, the EFSA GMO Panel recommends the applicant update it accordingly, by following the same aforementioned methodology and reporting policy.

OVERALL CONCLUSIONS AND RECOMMENDATIONS

No new data on the single maize events Bt11, MIR162, MIR604 and GA21 that would lead to a modification of the original conclusions on their safety were identified.

The combination of maize events Bt11, MIR162, MIR604 and GA21 in the four-event stack maize Bt11 × MIR162 × MIR604 × GA21 did not give rise to issues – relating to molecular, agronomic, phenotypic or compositional characteristics – regarding food and feed safety.

The newly expressed proteins in the four-event stack maize did not raise concerns for human and animal health. The compositional data indicate that maize Bt11 × MIR162 × MIR604 × GA21 would be expected to deliver the same nutrition as its conventional counterpart. This was confirmed by the results of an animal feeding study in chickens for fattening.

The EFSA GMO Panel considers that there is no reason to expect interactions that could impact on food and feed safety. No safety concerns are foreseen for any subcombinations of the individual events, including those not previously assessed by EFSA.

Considering the introduced traits and the outcome of the comparative analysis, the routes of exposure and the limited exposure levels, the EFSA GMO Panel concluded that this four-event stack maize would not raise safety concerns in case of accidental release of viable GM maize grains into the environment, irrespective of possible interactions between the individual events within this four-event stack maize. Moreover, in the light of the scope of the application, the data available for various subcombinations and the poor ability of maize to survive outside cultivated land, the EFSA GMO Panel is of the opinion that any subcombinations of the individual events, including those not previously assessed by EFSA, would not raise environmental safety concerns.

Post-market monitoring of food/feed derived from maize Bt11 × MIR162 × MIR604 × GA21 or the 10 subcombinations is not considered necessary.

The PMEM plan submitted by the applicant for the four-event stack maize does not include any provision for the six subcombinations that were not previously assessed. Therefore, the EFSA GMO Panel recommends the applicant to revise the plan accordingly.

RECOMMENDATION

The EFSA GMO Panel did not find indication that the subcombinations, resulting from combination of any of the single events included in the four-stack, would raise safety concerns. However, for some

[47] Dossier: Part I – Section D11 and Appendix 35.
[48] EFSA-GMO-UK-2007-48, EFSA-GMO-UK-2007-49, EFSA-GMO-UK-2007-50, EFSA-GMO-UK-2007-56.

subcombinations (Bt11 x MIR162 x MIR604, MIR162 x MIR604 x GA21, Bt11 x MIR162, MIR162 x MIR604, MIR162 x GA21) that could be produced by conventional crossing through targeted breeding approaches, little or no specific data were submitted. For these the EFSA GMO Panel has drawn conclusions on a weight-of-evidence approach, giving rise to uncertainties due to data gaps.

In order to reduce these uncertainties and to confirm assumptions made for the assessment of these subcombinations, the EFSA GMO Panel recommends that the applicant collate relevant information, if these subcombinations were to be created via targeted breeding approaches and commercialised in the future. In this case, this information should focus on expression levels of the newly expressed proteins.

CORRESPONDENCE

1. Letter from the Competent Authority of Germany, received on 20 February 2009, concerning a request for placing on the market of maize Bt11 × MIR162 × MIR604 × GA21 in accordance with Regulation (EC) No 1829/2003.

2. Acknowledgement letter, dated 4 March 2009, from EFSA to the Competent Authority of Germany.

3. Letter from EFSA to applicant, dated 27 March 2009, requesting additional information under completeness check.

4. Letter from applicant to EFSA, received on 19 May 2009, providing additional information under completeness check.

5. Letter from EFSA to applicant, dated 3 June 2009, requesting additional information under completeness check.

6. Letter from applicant to EFSA, received on 12 June 2009 then updated on 24 June 2009, providing additional information under completeness check.

7. Letter from EFSA to applicant, dated 13 July 2009, delivering the 'Statement of Validity' for application EFSA-GMO-DE-2009-66, maize Bt11 × MIR162 × MIR604 × GA21 submitted by Syngenta under Regulation (EC) No 1829/2003.

8. Letter from EFSA to applicant, dated 21 September 2009, requesting additional information and stopping the clock.

9. Letter from applicant to EFSA, received on 21 December 2009, providing additional information.

10. Letter from EFSA to applicant, dated 5 February 2010, requesting additional information.

11. Letter from applicant to EFSA, received on 5 October 2010, providing additional information.

12. Letter from EFSA to applicant, dated 17 March 2010, requesting additional information.

13. Letter from applicant to EFSA, received on 3 June 2010, providing additional information.

14. Letter from applicant to DG Health and Consumer Protection of the European Commission, dated 14 June 2010, clarifying the scope of the application.

15. Letter from EFSA to applicant, received on 15 September 2010, clarifying that "*a risk assessment of the single events is a pre-requisite for the risk assessment of stacked events*" and maintaining the clock stopped.

16. Letter from EFSA to applicant, dated 21 January 2011, requesting additional information.

17. Letter from applicant to EFSA, received on 1 February 2012, providing additional information
18. Letter from applicant to EFSA, dated 15 March 2012, clarifying the scope of the application.
19. Letter from EFSA to applicant, dated 6 June 2012, with the adoption of a scientific opinion on maize MIR162, restarting the clock.
20. Letter from EFSA to applicant, dated 6 June 2012, confirming the scope of the application.
21. Letter from EFSA to applicant, dated 6 July 2012, requesting additional information.
22. Letter from applicant to EFSA, received on 10 October 2012, providing additional information.
23. Letter from EFSA to applicant, dated 7 December 2012, requesting additional information.
24. Letter from applicant to EFSA, received on 19 March 2013, providing additional information.
25. Letter from EFSA to applicant, dated 5 February 2013, requesting additional information.
26. Letter from applicant to EFSA, received on 25 March 2013, providing additional information.
27. Letter from applicant to EFSA, dated 8 July 2013, redefining the scope of the application.
28. Letter from applicant copy to EFSA, received on 24 July 2013, justifying the scope redefinition.
29. Letter from EFSA to the applicants, dated 27 September 2013, restarting the clock.
30. Letter from applicant to EFSA, received on 10 December 2013, spontaneously providing additional information.
31. Letter from EFSA to applicant, dated 5 February 2014, requesting additional information.
32. Letter from applicant to EFSA, received on 18 February 2014, providing additional information.
33. Letter from applicant to EFSA, received on 28 July 2014, spontaneously providing additional information.
34. Letter from EFSA to applicant, dated 9 September 2014, requesting additional information.
35. Letter from applicant to EFSA, received on 25 September 2014, providing additional information.
36. Letter from EFSA to applicant, dated 16 September 2014, requesting additional information.
37. Letter from applicant to EFSA, received on 15 October 2014, providing additional information
38. Letter from EFSA to applicant, dated 24 October 2014, requesting additional information.
39. Letter from applicant to EFSA, received on 3 July 2015, providing additional information.
40. Letter from EFSA to applicant, dated 21 July 2015, spontaneously providing additional information.
41. Letter from EFSA to applicant, dated 10 August 2015, spontaneously providing additional information.
42. Letter from EFSA to applicant, dated 18 September 2015, requesting additional information.

43. Letter from applicant to EFSA, received on 24 September 2015, providing additional information.

44. Letter from EFSA to the applicants, dated 23 October 2015, restarting the clock.

REFERENCES

Bergamasco VB, Mendes DRP, Fernandes OA, Desidério JA and Lemos MVF, 2013. *Bacillus thuringiensis* Cry1Ia10 and Vip3Aa protein interactions and their toxicity in Spodoptera spp. (Lepidoptera). Journal of Invertebrate Pathology, 112, 152–158.

Chen E, and Stacy C, 2003. Modified Cry3A toxins having increased toxicity to corn rootworm, their nucleic acid sequences, and methods for controlling plant pests. Patent Cooperation Treaty International Publication Number WO 03/018810 A2, 127 pp.

CVB (Dutch Central Feed Bureau), 2001. Tabellenboek Veevoeding 2001. Voedernormen landbouwhuisdieren voederwaarde veevoeders. Cevtraal Veevoederbureau, Lelystad, the Netherlands.

CVB (Dutch Central Feed Bureau), 2002. Veevoedertabel 2002. Gegewens over chemische samestelling, verteerbaarheid en voederwaarde van voedermiddelen. Cevtraal Veevoederbureau, Lelystad, the Netherlands.

Codex Alimentarius, 2009. Foods derived from modern biotechnology. Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme. Rome, Italy, 85 pp.

Eastham K and Sweet J, 2002. Genetically modified organisms (GMOs): the significance of gene flow through pollen transfer. Environmental Issue Report No 28. European Environment Agency, Copenhagen, Denmark. Available online: http://www.eea.europa.eu/publications/environmental_issue_report_2002_28.

EFSA (European Food Safety Authority), 2005. Opinion of the Panel on Genetically Modified Organisms (GMO Panel) on a request from the Commission related to the notification (Reference C/F/96/05.10) for the placing on the market of insect-tolerant genetically modified maize Bt11, for cultivation, feed and industrial processing, under Part C of Directive 2001/18/EC from Syngenta Seeds. The EFSA Journal 2005, 213, 1–33.

EFSA (European Food Safety Authority), 2006. Guidance Document of the Panel on Genetically Modified Organisms (GMO Panel) for the risk assessment of genetically modified plants and derived food and feed. The EFSA Journal 2006, 99, 1–100.

EFSA (European Food Safety Authority), 2007a. Guidance document of the Panel on Genetically Modified Organisms (GMO Panel) on genetically modified organisms for the risk assessment of genetically modified plants containing stacked transformation events. The EFSA Journal 2007, 512, 1–5.

EFSA (European Food Safety Authority), 2007b. Opinion of the Panel on Genetically Modified Organisms (GMO Panel) on application (Reference EFSA-GMO-UK-2005-19 and EFSA-GMO-RX-GA21) for the placing on the market of glyphosate-tolerant genetically modified maize GA21, for food and feed uses, import and processing and for renewal of the authorisation of maize GA21 as existing product, both under Regulation (EC) No 1829/2003 from Syngenta Seeds S.A.S. on behalf of Syngenta Crop Protection AG. The EFSA Journal 2007, 541, 1–25.

EFSA (European Food Safety Authority), 2009a. Opinion of the Panel on Genetically Modified Organisms (GMO Panel) on application reference EFSA-GMO-RX-Bt11 for renewal of the authorisation of existing products produced from insect-resistant genetically modified maize Bt11, under Regulation (EC) No 1829/2003 from Syngenta. The EFSA Journal 2009, 977, 1–13.

EFSA (European Food Safety Authority), 2009b. Scientific Opinion of the Panel on Genetically Modified Organisms (GMO Panel) on application (Reference EFSA-GMO-UK-2005-11) for the placing on the market of insect-resistant genetically modified maize MIR604 event, for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Syngenta Seeds S.A.S on behalf of Syngenta Crop Protection AG. The EFSA Journal 2009, 1193, 1–26.

EFSA (European Food Safety Authority). 2009c. Bilateral technical meeting between members of the Panel on genetically modified organism and the VKM Norwegian delegation- Adjuvanticity of Cry proteins. EFSA/GMO/472, 1–2. Available online: http://www.efsa.europa.eu/en/gmo/gmomsmeetings.htm

EFSA Panel on Genetically Modified Organisms (GMO), 2009. Scientific Opinion on application (Reference EFSA-GMO-UK-2007-49) for the placing on the market of insect resistant and herbicide tolerant genetically modified maize Bt11xGA21, for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Syngenta Seeds. EFSA Journal 2009;7(9):1319, 27 pp. doi: 10.2903/j.efsa.2009.1319

EFSA Panel on Genetically Modified Organisms (GMO), 2010a. Scientific Opinion on application (EFSA-GMO-UK-2007-48) for the placing on the market of insect resistant and herbicide tolerant genetically modified maize MIR604 × GA21 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Syngenta Seeds. EFSA Journal 2010;8(5):1611, 30 pp. doi:10.2903/j.efsa.2010.1611

EFSA Panel on Genetically Modified Organisms (GMO), 2010b. Scientific Opinion on application (Reference EFSA-GMO-UK-2007-50) for the placing on the market of insect resistant and herbicide tolerant genetically modified maize Bt11xMIR604, for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Syngenta Seeds. EFSA Journal 2010;8(5):1614, 30 pp. doi: 10.2903/j.efsa.2010.1614

EFSA Panel on Genetically Modified Organisms (GMO), 2010c. Scientific Opinion on application (Reference EFSA-GMO-UK-2008-56) for the placing on the market of insect resistant and herbicide tolerant genetically modified maize Bt11 × MIR604 × GA21, for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Syngenta Seeds. EFSA Journal 2010;8(5):1616, 30 pp. doi: 10.2903/j.efsa.2010.1616

EFSA Panel on Genetically Modified Organisms (GMO), 2011a. Guidance for risk assessment of food and feed from genetically modified plants. EFSA Journal 2011;9(5):2150, 37 pp. doi:10.2903/j.efsa.2011.2150

EFSA Panel on Genetically Modified Organisms (GMO), 2011b. Guidance on the post-market environmental monitoring (PMEM) of genetically modified plants. EFSA Journal 2011;9(8):2316, 40 pp. doi:10.2903/j.efsa.2011.2316

EFSA Panel on Genetically Modified Organisms (GMO), 2012a. Scientific Opinion on application (EFSA-GMO-DE-2010-82) for the placing on the market of insect-resistant genetically modified maize MIR162 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Syngenta. EFSA Journal 2012;10(6):2756, 27 pp. doi:10.2903/j.efsa.2012.2756

EFSA Panel on Genetically Modified Organisms (GMO), 2012b. Scientific Opinion updating the risk assessment conclusions and risk management recommendations on the genetically modified insect resistant maize Bt11. EFSA Journal 2012;10(12):3018, 104 pp. doi:10.2903/j.efsa.2012.3018

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2013. Scientific opinion on applications EFSA-GMO-RX-T25 and EFSA-GMO-NL-2007-46 for the renewal of authorisation of maize T25,1 and for the placing on the market of herbicide-tolerant genetically modified maize T25,2 both for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Bayer CropScience AG. EFSA Journal 2013;11(10):3356, 30 pp. doi:10.2903/j.efsa.2013.3356

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2015a. Statement on the risk assessment of new sequencing data on GM maize event MIR604. EFSA Journal 2015;13(10):4255, 6 pp. doi:10.2903/j.efsa.2015.4255

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2015b. Statement on the risk assessment of new sequencing data on GM maize event GA21. EFSA Journal 2015;13(11):4296

Estruch JJ, Warren GW, Mullins MA, Nye GJ, Craig JA and Koziel MG, 1996. Vip3A, a novel Bacillus thuringiensis vegetative insecticidal protein with a wide spectrum of activities against

lepidopteran insects. Proceedings of the National Academy of Sciences of the United States of America, 93, 5389–5394.

Einspanier R, Lutz B, Rief S, Berezina O, Zverlov V, Schwarz W and Mayer J, 2004. Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgenic maize. European Food Research and Technology, 218, 269–273.

Fang J, Xu X, Wang P, Zhao JZ, Shelton AM, Cheng J, Feng MG and Shen Z, 2007. Characterization of chimeric *Bacillus thuringiensis* Vip3 toxins. Applied and Environmental Microbiology, 73, 956–961.

Gruber S, Colbach N, Barbottin A and Pekrun C, 2008. Post-harvest gene escape and approaches for minimizing it. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, 3, 1–17.

Guertler P, Lutz B, Kuehn R, Meyer HHD, Einspanier R, Killermann B and Albrecht C, 2008. Fate of recombinant DNA and Cry1Ab protein after ingestion and dispersal of genetically modified maize in comparison to rapeseed by fallow deer (*Dama dama*). European Journal of Wildlife Research, 54, 36–43.

Hammond B, Kough J, Herouet-Guicheney C, Jez JM; on behalf of the ILSI International Food Biotechnology Committee Task Force on the Use of Mammalian Toxicology Studies in the Safety Assessment of GM Foods, 2013. Toxicological evaluation of proteins introduced into food crops. Critical Reviews in Toxicology, 43(Suppl 2), 25–42.

Herrmann KM, 1995. The Shikimate Pathway: early steps in the biosynthesis of aromatic compounds. Plant Cell, 7(7), 907–919.

Icoz I and Stotzky G, 2008. Fate and effects of insect-resistant Bt crops in soil ecosystems. Soil Biology and Biochemistry, 40, 559–586.

Kuiper HA, Kleter GA, Noteborn HPJM and Kok EJ, 2001. Assessment of the food safety issues related to genetically modified foods. The Plant Journal, 27, 503–528.

Lebrun M, Sailland A, Freyssinet G and Degryse E, 2003. Mutated 5-enoylpyruvylshikimate-3-phosphate synthase, gene coding for said protein and transformed plants containing said gene. Bayer CropScience S.A. (Lyons, FR) Patent No 6,566,587.

Lecoq E, Holt K, Janssens J, Legris G, Pleysier A, Tinland B and Wandelt C, 2007. General surveillance: roles and responsibilities the industry view. Journal of Consumer Protection and Food Safety, 2(S1), 25–28.

Lee MK, Walters FS, Hart H, Palekar N and Chen JS, 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab δ-Endotoxin. Applied and Environmental Microbiology, 69, 4648–4657.

Lee MK, Miles P and Chen JS, 2006. Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. Biochemical and Biophysical Research Communications, 339, 1043–1047.

Lutz B, Wiedemann S, Einspanier R, Mayer J and Albrecht C, 2005. Degradation of Cry1Ab protein from genetically modified maize in the bovine gastrointestinal tract. Journal of Agricultural and Food Chemistry, 53, 1453–1456.

Lutz B, Wiedemann S and Albrecht C, 2006. Degradation of transgenic Cry1Ab DNA and protein in Bt-176 maize during the ensiling process. Journal of Animal Physiology and Animal Nutrition (Berlin), 90, 116–123.

Markovitz A, Sydiskis RJ and Lieberman MM, 1967. Genetic and biochemical studies on mannose-negative mutants that are deficient in phosphomannose isomerase in *Escherichia coli* K-12. Journal of Bacteriology, 94, 1492–1496.

Moreno-Fierros L, Ruiz-Medina EJ and Esquivel R, López-Revilla R and Piña-Cruz S, (2003) Intranasal Cry1Ac protoxin is an effective mucosal and systemic carrier and adjuvant of

Streptococcus pneumoniae polysaccharides in mice. Scandinavian Journal of Immunology. 57: 45-55.

Negrotto D, Jolley M, Beer S, Wenck AR and Hansen G, 2000. The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. Plant Cell Reports, 19, 798–803.

Noteborn HPJM, Bienenmann-Ploum ME, Van Den Berg JHJ, Alink GM, Zolla L, Reynaerts A, Pensa M and Kuiper HA, 1995. Safety assessment of the *Bacillus thuringiensis* insecticidal crystal protein CRYIA(b) expressed in transgenic tomatoes. In: Genetically modified foods – safety aspects, ACS Symposium Series 605. Eds Engel K-H, Takeoka GR and Teranishi R. American Chemical Society, Washington, DC, USA, 134–147.

NRC (National Research Council), 1994. Nutrient Requirements of Poultry, 9th Revised Edition. National Academy Press, Washington, DC, USA.

OECD (Organisation for Economic Co-operation and Development), 2002. Consensus Document on compositional considerations for new varieties of maize (*Zea mays*): key food and feed nutrients, anti-nutrients and secondary plant metabolites. Series on the Safety of Novel Food and Feeds, No 6, 1–42.

OECD (Organisation for Economic Co-operation and Development), 2003. Consensus Document on the biology of *Zea mays* subsp. Mays (Maize). Series on Harmonisation of Regulatory Oversight in Biotechnology (ENV/JM/MONO(2003)11), No 27, 1–49.

Palaudelmàs M, Peñas G, Melé E, Serra J, Salvia J, Pla M, Nadal A and Messeguer J, 2009. Effect of volunteers on maize gene flow. Transgenic Research, 18, 583–594.

Paul V, Guertler P, Wiedemann S and Meyer HHD, 2010. Degradation of Cry1Ab protein from genetically modified maize (MON810) in relation to total dietary feed proteins in dairy cow digestion. Transgenic Research, 19, 683–689.

Reynolds TL, Nemeth MA, Glenn KC, Ridley WP and Astwood JD, 2005. Natural variability of metabolites in maize grain: differences due to genetic background. Journal of Agricultural and Food Chemistry, 53, 10061–10067.

Rojas-Hernandez S, Rodriguez-Monroy MA, Lopez-Revilla R, Resendiz-Albor AA and Moreno-Fierros L, 2004. Intranasal co-administration of the Cry1Ac protoxin with amoebal lysates increases protection against *Naegleria fowleri* meningoencephalitis. Infection and Immunity, 72, 4368–4375.

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR and Dean DH, 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiology and Molecular Biology Reviews, 62, 775–806.

Sekar V, Thompson DV, Maroney MJ, Bookland RG and Adang MJ, 1987. Molecular cloning and characterization of the insecticidal crystal protein gene of *Bacillus thuringiensis* var. *tenebrionis*. Proceedings of the National Academy of Sciences of the United States of America, 84, 7036–7040.

Vázquez-Padrón RI, Moreno Fierros L, Neri Bazán L, de la Riva GA and López Revilla R, 1999. Intragastric and intraperitoneal administration of Cry1Ac protoxin from *Bacillus thuringiensis* induce systemic and mucosal immune response in mice. Life Sciences, 64, 1897–1912.

Wiedemann S, Lutz B, Kurtz H, Schwarz FJ and Albrecht C, 2006. *In situ* studies on the time-dependent degradation of recombinant corn DNA and protein in the bovine rumen. Journal of Animal Science, 84, 135–144.

Windels P, Alcalde E, Lecoq E, Legris G, Pleysier A, Tinland B and Wandelt C, 2008. General surveillance for import and processing: the EuropaBio approach. Journal of Consumer Protection and Food Safety, 3(S2), 14–16.

Wohlleben W, Arnold W, Broer I, Hillemann D, Strauch E and Pühler A, 1988. Nucleotide sequence of the phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. Gene, 70, 25–37.

APPENDIX

Appendix A. Summary of experimental data provided in previously assessed applications

Risk assessment area	Maize events							
	Bt11	**MIR162**	**MIR604**	**GA21**	**Bt11 × GA21**	**Bt11 × MIR604**	**MIR604 × GA21**	**Bt11 × GA21 × MIR604**
Newly expressed proteins	Cry1Ab, PAT[(a)]	Vip3Aa20 PMI[(a)]	mCry3A, PMI[(a)]	mEPSPS	Cry1Ab, PAT[(a)], mEPSPS,	Cry1Ab, PAT[(a)], mCry3A, PMI[(a)]	mCry3A, PMI[(a)], mEPSPS	Cry1Ab, PAT[(a)], mEPSPS, mCry3A, PMI[(a)]
Molecular characterisation								
Transformation process and vector constructs	√	√	√	√				
Insert structure and flanking regions	√	√	√	√				
Bioinformatic searches	√	√	√	√	√	√	√	√
Integrity and genetic stability of the insert(s)	√	√	√	√	√	√	√	√
Protein expression	√	√	√	√	√	√	√	√
Comparative assessment								
Field trials	√	√	√	√	√	#	#	√
Agronomic and phenotypic characteristics	√	√	√	√	√	#	#	√
Compositional analysis	√	√	√	√	√	#	#	√
Food and feed safety								
Characterisation of the newly expressed protein(s)	√	√	√	√				
Heat stability	√	√	√	√				
Degradation in simulated digestive fluids	√	√	√	√				
Acute toxicity study with newly expressed proteins	√	√	√	√				
Repeated-dose toxicity study with newly expressed proteins		√						
Rodent feeding study with whole food and feed	#	√	√	√				
Feeding study in fast-growing animals (e.g., broiler)	√	√	√	√	√	√	√	√
Feeding study in farm animals (e.g., calves, pig, cows, sheeps, etc)	√							
Environmental risk assessment								
Pollen viability	√	√	√	√	√	√	√	√
Seed germination	√	√	√	√	√	√	√	√

(a): Selectable markers, and PMI expressed in MIR162 differs from the one expressed in MIR604.

√, Data were generated for the event in question, blank cells indicate that no data were provided; #, data not specific to the event in question, e.g. a subchronic toxicity study was performed with feed formulated from the Bt11 maize grain, but from another maize event expressing the Cry1Ab protein.