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Assessment of genetically modified maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-DE-2011-103)

EFSA Panel on Genetically Modified Organisms (EFSA GMO Panel), Hanspeter Naegeli, Jean-Louis Bresson, Tamas Dalmay, Ian Crawford Dewhurst, Michelle M Epstein, Leslie George Firbank, Philippe Guerche, Jan Hejatko, Francisco Javier Moreno, Ewen Mullins, Fabien Nogué, Nils Rostoks, Jose Juan Sánchez Serrano, Giovanni Savoini, Eve Veromann, Fabio Veronesi, Michele Ardizzone, Fernando Álvarez, Antonio Fernandez Dumont, Andrea Gennaro, Anna Lanzoni, Franco Maria Neri, Nikoletta Papadopoulou, Konstantinos Paraskevopoulos, Giacomo De Sanctis, Tommaso Raffaello, Silvia Federici and Marina Koukoulanaki

Abstract

Maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 (six-event stack maize) was produced by conventional crossing to combine six single events: Bt11, MIR162, MIR604, 1507, 5307 and GA21. The GMO Panel previously assessed the six single events and 22 of their combinations and did not identify safety concerns. No new data on the maize single events or their 22 combinations that could lead to modification of the original conclusions on their safety have been identified. The molecular characterisation, comparative analysis (agronomic, phenotypic and compositional characteristics) and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single maize events and of the newly expressed proteins in the six–event stack maize does not give rise to food and feed safety and nutritional concerns. The GMO Panel concludes that the six-event stack maize, as described in this application, is as safe as and nutritionally equivalent to its non-GM comparator and the non-GM reference varieties tested. In the case of accidental release of viable grains of the six-event stack maize into the environment, this would not raise environmental safety concerns. The GMO Panel assessed the likelihood of interactions among the single events in the 34 maize subcombinations not previously assessed and concludes that these are expected to be as safe as and nutritionally equivalent to the single events, the previously assessed subcombinations and the six-event stack maize. The post-market environmental monitoring plan and reporting intervals are in line with the intended uses of the six-event stack maize. Post-market monitoring of food/feed is not considered necessary. The GMO Panel concludes that the six-event stack maize and its subcombinations are as safe as its non-GM comparator and the tested non-GM reference varieties with respect to potential effects on human and animal health and the environment.

Keywords: GMO, maize (*Zea mays*), Bt11, MIR162, MIR604, 1507, 5307, GA21, herbicide tolerance, insect resistance, Regulation (EC) No 1829/2003

Requestor Competent Authority of Germany

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Correspondence: GMO_Secretariat_applications@efsa.europa.eu

Panel members: Jean-Louis Bresson, Tamas Dalmay, Ian Crawford Dewhurst, Michelle M Epstein, Leslie George Firbank, Philippe Guerche, Jan Hejatko, Francisco Javier Moreno, Ewen Mullins, Hanspeter Naegeli, Fabien Nogué, Nils Rostoks, Jose Juan Sánchez Serrano, Giovanni Savoini, Eve Veromann and Fabio Veronesi.

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Summary

Following the submission of application EFSA-GMO-DE-2011-103 under Regulation (EC) No 1829/2003 from Syngenta, the Panel on Genetically Modified Organisms of the European Food Safety Authority (hereafter referred to as the 'GMO Panel') was asked to deliver a scientific opinion on genetically modified (GM) maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 (hereafter referred to as 'the six-event stack maize') and its subcombinations independently of their origin (referred to hereafter as 'subcombinations'). The scope of application EFSA-GMO-DE–2011-103 is for the placing on the market of maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 and all its subcombinations independently of their origin for food and feed uses, import and processing.

The term 'subcombination' refers to any combination of up to five of the events present in the six-event stack maize. The safety of subcombinations occurring as segregating progeny in the harvested grains of maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 is evaluated in the context of the assessment of the six-event stack maize in Section 3.3 of the present GMO Panel scientific opinion. The safety of the subcombinations that either have been or could be produced by conventional crossing through targeted breeding approaches, which can be bred, produced and marketed independently of the six-event stack maize, are risk assessed in Section 3.4 of the present scientific opinion.

In delivering its scientific opinion, the GMO Panel considered the information available on the single events, the six-event stack maize, 25 of the subcombinations (10 two-event stacks, 10 three-event stacks, 4 four-event stacks and a five-event stack), the scientific comments submitted by the Member States and the relevant scientific literature. The six-event stack maize was produced by conventional crossing to combine six single maize events: Bt11 (expressing Cry1Ab and the phosphinothricin acetyl transferase (PAT) proteins); MIR162 (expressing the Vip3Aa20 and the phosphomannose isomerase (PMI) proteins); MIR604 (expressing a modified Cry3A (mCry3A) and the PMI proteins); 1507 (expressing the Cry1F and the PAT proteins); 5307 (expressing the eCry3.1Ab and the PMI proteins); and GA21 (expressing the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (mEPSPS)). Herbicide tolerance traits are achieved by the expression of mEPSPS protein from *Zea mays* and PAT protein from *Streptomyces viridochromogenes*. Insect resistance traits are achieved by the expression of the Cry1Ab, Vip3Aa20 and Cry1F proteins from *Bacillus thuringiensis* for protection against specific lepidopteran pests and by the expression of the mCry3A and eCry3.1Ab proteins from *B. thuringiensis* for protection against corn rootworm (*Diabrotica* spp.) larval feeding.

The GMO Panel evaluated the six-event stack maize and its subcombinations with reference to the scope and appropriate principles described in its guidelines for the risk assessment of GM plants and derived food and feed, the environmental risk assessment of GM plants and the post-market environmental monitoring (PMEM) of GM plants.

For application EFSA-GMO-DE-2011-103, previous assessments of the six single maize events Bt11, MIR162, MIR604, 1507, 5307 and GA21 and 22 of the subcombinations (10 two-event stacks, 9 three-event stacks and 3 four-event stacks), together with new information on three subcombinations, provided a basis to evaluate the six-event stack maize and all its subcombinations. No safety concerns were identified by the GMO Panel in the previous assessments. No safety issue concerning the six single maize events was identified by the updated bioinformatic analyses, nor reported by the applicant since the publication of the previous GMO Panel scientific opinions. Therefore, the GMO Panel considers that its previous conclusions on the safety of the single maize events remain valid.

For the six-event stack maize, the risk assessment included the molecular characterisation of the inserted DNA and analysis of protein expression. An evaluation of the comparative analysis of agronomic/phenotypic and compositional characteristics was undertaken, and the safety of the newly expressed proteins and the whole food and feed were evaluated with respect to potential toxicity, allergenicity and nutritional characteristics. An evaluation of environmental impacts and the PMEM plan was also undertaken.

The molecular characterisation data establish that the events stacked in maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 have retained their integrity. Protein expression analyses show that the levels of the newly expressed proteins are similar in the six-event stack and in the single events or already assessed subcombinations, except for the expected higher levels of the PAT and PMI proteins in the six-event stack resulting from the combination of events Bt11 and 1507 (both producing PAT) and events MIR162, 5307 and MIR604 (all producing PMI). No indications of interactions that may affect the integrity of the events and the levels of the newly expressed proteins in this six-event stack maize are identified.

The comparative analysis of forage and grain composition and agronomic/phenotypic characteristics identified no differences between maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 and the non-GM comparator that required further assessment for food/feed safety or environmental impact, except for the levels of ash, potassium, zinc, β-carotene, folic acid, methionine, arachidic acid (C20:0) and ferulic acid in grain and for the agronomic/phenotypic endpoints early stand count, final stand count, days to 50% pollen shed and grain moisture. All those changes were further assessed and not found to have a safety impact.

The molecular characterisation, the comparative analysis and the outcome of the toxicological, allergenicity and nutritional assessment indicate that the combination of the single maize events and of the newly expressed proteins in the six-event stack maize does not give rise to food and feed safety and nutritional concerns. The GMO Panel concludes that maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21, as described in this application, is as safe as and nutritionally equivalent to its non-GM comparator and the non-GM reference varieties tested.

Considering the events combined and their potential interactions, the outcome of the comparative analysis and the routes and levels of exposure, the GMO Panel concludes that maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 would not raise safety concerns in the case of accidental release of viable GM maize grains into the environment.

Since no new safety concerns were identified for the 22 previously assessed subcombinations (10 two event stacks, 9 three-event stacks and 3 four-event stacks) and no new data leading to the modification of the original conclusions on safety were identified, the GMO Panel considers that its previous conclusions on these maize subcombinations remain valid. The remaining 34 subcombinations included in the scope of application EFSA-GMO-DE-2011-103 have not been previously assessed; for three of them, the applicant provided new information that was considered by the GMO Panel. The GMO Panel assessed the possibility of interactions between the events in the 34 subcombinations and concludes that these combinations would not raise safety concerns. These subcombinations are therefore expected to be as safe as and nutritionally equivalent to the single events, the previously assessed subcombinations and the six-event stack maize.

Given the absence of safety concerns for foods and feeds from maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 and all its subcombinations, the GMO Panel considers that post-market monitoring of these products is not necessary. The PMEM plan and reporting intervals are in line with the intended uses of the six-event stack maize and its subcombinations.

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1. Introduction

The scope of application EFSA-GMO-DE-2011-103 is for food and feed uses, import and processing in the European Union (EU) of the genetically modified (GM) herbicide-tolerant insect-resistant maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 and all its subcombinations independently of their origin.

1.1. Background

On 16 December 2011, the European Food Safety Authority (EFSA) received from the Competent Authority of Germany application EFSA-GMO-DE-2011-103 for authorisation of maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 (hereafter referred to as 'the six-event stack maize') (Unique Identifier: SYNBTØ111 × SYNIR162-4 × SYNIR6Ø4-5 × DAS-Ø15Ø7-1 × SYN-Ø53Ø7-1 × MON-ØØØ21-9), submitted by Syngenta Crop Protection AG (hereafter referred to as 'the applicant') according to Regulation (EC) No 1829/2003[1].

Following receipt of application EFSA-GMO-DE-2011-103, EFSA informed the Member States (MS) and the European Commission and made the summary of the application available to the public on the EFSA website.[2]

EFSA checked the application for compliance with the relevant requirements of Regulation (EC) No 1829/2003 and, when needed, asked the applicant to supplement the initial application. On 18 August 2014, EFSA declared the application valid and made the valid application available to MS and the European Commission.

From the validity date, EFSA and its scientific Panel on Genetically Modified Organisms (hereafter referred to as 'the GMO Panel') endeavoured to respect a time limit of 6 months to issue a scientific opinion on application EFSA-GMO-DE-2011-103. Such time limit was extended whenever EFSA and/or its GMO Panel requested supplementary information to the applicant. According to Regulation (EC) No 1829/2003, any supplementary information provided by the applicant during the risk assessment was made available to the EU MS and European Commission (for further details, see the section 'Documentation', below).

In accordance with Regulation (EC) No 1829/2003, EFSA consulted the nominated risk assessment bodies of EU MS, including national Competent Authorities within the meaning of Directive 2001/18/EC[3]. The EU MS had 3 months to make their opinion known on application EFSA-GMO-DE-2011-103 as of date of validity.

1.2. Terms of Reference as provided by the requestor

According to Articles 6 and 18 of Regulation (EC) No 1829/2003, EFSA and its GMO Panel were requested to carry out a scientific risk assessment of the six-event stack maize and all its subcombinations independently of their origin, for food and feed uses, import and processing.

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

In addition to the present scientific opinion, EFSA was also asked to report on the particulars listed under Articles 6(5) and 18(5) of Regulation (EC) No 1829/2003, but not to give an opinion on them, because they pertain to risk management.[4]

2. Data and methodologies

2.1. Data

The GMO Panel based its scientific assessment of the six-event stack maize on the valid application EFSA-GMO-DE-2011-103, additional information provided by the applicant during the risk assessment, scientific comments submitted by MS and relevant peer-reviewed scientific publications.

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

[2] Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2012-00069

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

[4] These particulars can be found in the technical report by EFSA on application EFSA-GMO-DE-2011-103, made available in the EFSA Register of Questions.

2.2. Methodologies

The GMO Panel conducted its assessment in line with the principles described in Regulation (EU) No 1829/2003, its applicable guidelines (EFSA GMO Panel, 2010a, 2011a,b) and explanatory notes (EFSA, 2017a,b) for the risk assessment of GM plants.

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

3. Assessment

3.1. Introduction

Application EFSA-GMO-DE-2011-103 covers the six-event stack maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 and all its 56 subcombinations independently of their origin (Table 1).

The term 'subcombination' refers to any combination of up to five of the maize events Bt11, MIR162, MIR604, 1507, 5307 and GA21.

The safety of subcombinations occurring as segregating progeny in harvested grains of the six-event stack maize is evaluated in the context of the assessment of the six-event stack maize in Section 3.3 of the present scientific opinion.

'Subcombination' also covers combinations that have either been or could be produced by conventional crossing through targeted breeding approaches (EFSA GMO Panel, 2011a). These are maize stacks that can be bred, produced and marketed independently of the six-event stack maize. These subcombinations are assessed in Section 3.4 of this scientific opinion.

The six-event stack maize was produced by conventional crossing to combine six single maize events: Bt11 (expressing the proteins Cry1Ab and phosphinothricin acetyl transferase (PAT)), MIR162 (expressing the proteins Vip3Aa20 and phosphomannose isomerase (PMI)), MIR604 (expressing PMI and a modified Cry3A (mCry3A) protein), 1507 (expressing the proteins Cry1F and PAT), 5307 (expressing the proteins eCry3.1Ab and PMI) and GA21 (expressing the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (mEPSPS)).

Herbicide tolerance traits are achieved by the expression of the mEPSPS protein from *Zea mays* and PAT protein from *Streptomyces viridochromogenes*. Insect resistance traits are achieved by the expression of the Cry1Ab, Vip3Aa20 and Cry1F proteins from *Bacillus thuringiensis* for protection against specific lepidopteran pests, and the mCry3A and eCry3.1Ab proteins from *B. thuringiensis* for protection against corn rootworm (*Diabrotica* spp.) larval feeding.

Table 1: Stacked maize events covered by the scope of application EFSA-GMO-DE-2011-103

Degree of Stacking	Events
Six-event stack maize	Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21
Five-event stack maize	Bt11 × MIR162 × MIR604 × 1507 × 5307
	Bt11 × MIR162 × MIR604 × 1507 × GA21
	Bt11 × MIR162 × MIR604 × 5307 × GA21
	Bt11 × MIR162 × 1507 × 5307 × GA21
	Bt11 × MIR604 × 1507 × 5307 × GA21
	MIR162 × MIR604 × 1507 × 5307 × GA21
Four-event stack maize	Bt11 × MIR162 × MIR604 × 1507
	Bt11 × MIR162 × MIR604 × 5307
	Bt11 × MIR162 × MIR604 × GA21
	Bt11 × MIR604 × 1507 × 5307
	Bt11 × MIR604 × 1507 × GA21
	Bt11 × MIR162 × 1507 × 5307
	Bt11 × MIR162 × 1507 × GA21
	Bt11 × MIR162 × 5307 × GA21
	Bt11 × MIR604 × 5307 × GA21

Degree of Stacking	Events
	Bt11 × 1507 × 5307 × GA21
	MIR162 × MIR604 × 1507 × 5307
	MIR162 × MIR604 × 1507 × GA21
	MIR162 × MIR604 × 5307 × GA21
	MIR162 × 1507 × 5307 × GA21
	MIR604 × 1507 × 5307 × GA21
Three-event stack maize	Bt11 × MIR162 × MIR604
	Bt11 × MIR162 × 1507
	Bt11 × MIR162 × 5307
	Bt11 × MIR162 × GA21
	Bt11 × MIR604 × 1507
	Bt11 × MIR604 × 5307
	Bt11 × MIR604 × GA21
	Bt11 × 1507 × 5307
	Bt11 × 1507 × GA21
	Bt11 × 5307 × GA21
	MIR162 × MIR604 × 1507
	MIR162 × MIR604 × 5307
	MIR162 × MIR604 × GA21
	MIR162 × 1507 × 5307
	MIR162 × 1507 × GA21
	MIR162 × 5307 × GA21
	MIR604 × 1507 × 5307
	MIR604 × 1507 × GA21
	MIR604 × 5307 × GA21
	1507 × 5307 × GA21
Two-event stack maize	Bt11 × MIR162
	Bt11 × MIR604
	Bt11 × 1507
	Bt11 × 5307
	Bt11 × GA21
	MIR162 × MIR604
	MIR162 × 1507
	MIR162 × 5307
	MIR162 × GA21
	MIR604 × 1507
	MIR604 × 5307
	MIR604 × GA21
	1507 × 5307
	1507 × GA21
	5307 × GA21

All six single maize events and 22 of the subcombinations (10 two-event stacks, 9 three-event stacks and 3 four-event stacks) have been previously assessed (see Table 2). No concerns for human and animal health or environmental safety were identified.

Table 2: Single maize events and subcombinations of maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 previously assessed by the GMO Panel

Event	Application or mandate	EFSA Scientific Opinions
Bt11	C/F/96/05.10	EFSA (2005a)
	EFSA-GMO-RX-Bt11	EFSA (2009a)
	EFSA-M-2012–0232[a]	EFSA GMO Panel (2012a)
MIR162	EFSA-GMO-DE-2010-82	EFSA GMO Panel (2012b)
MIR604	EFSA-GMO-UK-2005-11	EFSA (2009b)
1507	C/NL/00/10	EFSA (2004)
	C/ES/01/01	EFSA (2005b)
	EFSA-GMO-NL-2004-02	EFSA (2005c)
	EFSA-GMO-RX-1507	EFSA (2009c)
	EFSA-M-2012-0231[b]	EFSA GMO Panel (2012c)
	EFSA-GMO-RX-001	EFSA GMO Panel (2017a)
5307	EFSA-GMO-DE-2011-95	EFSA GMO Panel (2015a)
	EFSA-M-2017-0011[c]	EFSA GMO Panel (2018a)
GA21	EFSA-GMO-UK-2005-19	EFSA (2007)
	EFSA-GMO-RX-GA21	
	EFSA-GMO-UK-2008-60	EFSA GMO Panel (2011c)
	EFSA-GMO-RX-005	EFSA GMO Panel (2017b)
Bt11 × MIR162	EFSA-GMO-DE-2009-66 EFSA-M-2016-0248[d]	EFSA GMO Panel (2015b) EFSA GMO Panel (2017c)
Bt11 × MIR604	EFSA-GMO-UK-2007-50	EFSA GMO Panel (2010b)
Bt11 × 1507	EFSA-GMO-DE-2011-99	EFSA GMO Panel (2016)
Bt11 × GA21	EFSA-GMO-UK-2007-49	EFSA GMO Panel (2009)
MIR162 × MIR604	EFSA-GMO-DE-2009-66	EFSA GMO Panel (2015b)
MIR162 × 1507	EFSA-GMO-DE-2010-86	EFSA GMO Panel (2018b)
MIR162 × GA21	EFSA-GMO-DE-2009-66	EFSA GMO Panel (2015b)
MIR604 × 1507	EFSA-GMO-DE-2011-99	EFSA GMO Panel (2016)
MIR604 × GA21	EFSA-GMO-UK-2007-48	EFSA GMO Panel (2010c)
1507 × GA21	EFSA-GMO-DE-2011-99	EFSA GMO Panel (2016)
Bt11 × MIR162 × MIR604	EFSA-GMO-DE-2009-66	EFSA GMO Panel (2015b)
Bt11 × MIR162 × 1507	EFSA-GMO-DE-2010-86	EFSA GMO Panel (2018b)
Bt11 × MIR162 × GA21	EFSA-GMO-DE-2009-66	EFSA GMO Panel (2015b)
Bt11 × MIR604 × GA21	EFSA-GMO-UK-2008-56	EFSA GMO Panel (2010d)
Bt11 × MIR604 × 1507	EFSA-GMO-DE-2011-99	EFSA GMO Panel (2016)
Bt11 × 1507 × GA21	EFSA-GMO-DE-2011-99 EFSA-M-2017-0169[e]	EFSA GMO Panel (2016) EFSA GMO Panel (2017d)
MIR162 × MIR604 × GA21	EFSA-GMO-DE-2009-66	EFSA GMO Panel (2015b)
MIR162 × 1507 × GA21	EFSA-GMO-DE-2010-86	EFSA GMO Panel (2018b)
MIR604 × 1507 × GA21	EFSA-GMO-DE-2011-99	EFSA GMO Panel (2016)
Bt11 × MIR162 × MIR604 × GA21	EFSA-GMO-DE-2009-66	EFSA GMO Panel (2015b)
Bt11 × MIR162 × 1507 × GA21	EFSA-GMO-DE-2010-86	EFSA GMO Panel (2018b)
Bt11 × MIR604 × 1507 × GA21	EFSA-GMO-DE-2011-99	EFSA GMO Panel (2016)

(a): Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2012-00713
(b): Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2012-00712
(c): Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2017-00052
(d): Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2016-00730
(e): Available online: http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2017-00669

3.2. Updated information on the single events[5]

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

The applicant clarified that the maize 1507 sequence reported in this application is identical to the corrected maize 1507 sequence (EFSA GMO Panel, 2017a). Analysis of the corrected sequencing data and the bioinformatic analyses performed on this sequence did not give rise to safety issues (EFSA GMO Panel, 2017a,e).

Updated bioinformatic analyses on the junction regions for maize events Bt11, MIR162, MIR604, 1507, 5307 and GA21, performed using the methodology specified in EFSA GMO Panel (2011a), confirm that no known endogenous genes were disrupted by any of the inserts.

Updated bioinformatic analyses of the amino acid sequence of the newly expressed Cry1F, PAT, Cry1Ab, Vip3Aa20, PMI, mCry3A, eCry3.1Ab and mEPSPS proteins reveal no significant similarities to toxins and allergens. Updated bioinformatic analyses of the newly created open reading frames (ORFs) within the insert or spanning the junctions between the insert and genomic DNA indicate that the expression of an ORF showing significant similarities to toxins or allergens is highly unlikely.

In order to assess the possibility for horizontal gene transfer (HGT) by homologous recombination, the applicant performed a sequence identity analysis with microbial DNA for maize events Bt11, MIR162, MIR604, 1507, 5307 and GA21. The likelihood and potential consequences of plant-to-bacteria gene transfer are described in Section 3.3.4.2.

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

3.3. Risk assessment of maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21

3.3.1. Molecular characterisation[6]

Possible interactions that would affect the integrity of the events, protein expression level or the biological function conferred by the individual inserts are considered below.

3.3.1.1. Genetics elements and their biological function

Maize events Bt11, MIR162, MIR604, 1507, 5307 and GA21 were combined by conventional crossing to produce the six-event stack maize. The structure of the inserts in the six-event stack is described in detail in the respective EFSA scientific opinions (Table 2) and no new genetic modifications were involved. Genetic elements in the expression cassettes of the single events are summarised in Table 3. Intended effects of the inserts in the six-event stack maize are summarised in Table 4.

Based on the known biological function of the newly expressed proteins (Table 4), the only foreseen interactions at the biological level are between the Vip3Aa20 protein and the four Cry proteins and between the four Cry proteins in susceptible insects.

[5] Dossier: Part II – Section A.2.2.2; additional information: 21/7/2015, 17/12/2015, 8/6/2018, 11/6/2018, 17/8/2018 and 8/2/2019.
[6] Dossier: Part II – Section A.2; additional information: 17/8/2018 and 19/12/2018.

Table 3: Genetic elements in the expression cassettes of the events stacked in maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21

Event	Promoter	5′ UTR	Transit peptide	Coding region*	Terminator
Bt11	35S (CaMV)	IVS6 (*Zea mays*)	–	*cry1Ab* (*Bacillus thuringiensis*)	*nos* (*Agrobacterium tumefaciens*)
	35S (CaMV)	IVS2 (*Z. mays*)	–	*pat* (*Streptomyces viridochromogenes*)	*nos* (*A. tumefaciens*)
MIR162	ZmUbiInt (*Z. mays*)	–	–	*vip3Aa20* (*B. thuringiensis*)	35S (CaMV)
	ZmUbiInt (*Z. mays*)	–	–	*pmi* (*Escherichia coli*)	*nos* (*A. tumefaciens*)
MIR604	MTL (*Z. mays*)	–	–	*mcry3A* (*B. thuringiensis*)	*nos* (*A. tumefaciens*)
	ZmUbiInt (*Z. mays*)	–	–	*Pmi* (*E. coli*)	*nos* (*A. tumefaciens*)
1507	Ubi1ZM (*Z. mays*)	–	–	*cry1F* (*B. thuringiensis*)	ORF25PolyA (*A. tumefaciens*)
	35S (CaMV)	–	–	*pat* (*S. viridochromogenes*)	35S (CaMV)
5307	CMP (CmYLCV)	–	–	*ecry3.1Ab* (*B. thuringiensis*)	*nos* (*A. tumefaciens*)
	ZmUbiInt (*Z. mays*)	–	–	*pmi* (*E. coli*)	*nos* (*A. tumefaciens*)
GA21	actin 1 (*Oryza sativa*)	actin 1 (*O. sativa*)	OTP (*Helianthus annuus*)	*mepsps* (*Z. mays*)	*nos* (*A. tumefaciens*)

*: Codon-optimised for expression in plants.
UTR: untranslated region; –: when no element was specifically introduced to optimise expression.

Table 4: Characteristics and intended effects of the events stacked in maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21

Event	Protein	Donor organism and biological function	Intended effects in GM plant
Bt11	Cry1Ab	Based on genes from *Bacillus thuringiensis* var. *kurstaki* HD-1. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal proteins (Cry) (Schnepf et al., 1998; Ellis et al., 2002)	Event Bt11 expresses a chimeric, truncated *cry1Ab* gene. Cry1Ab is a chimeric protein toxic to certain lepidopteran larvae feeding on maize
	PAT	Based on a gene from *Streptomyces viridochromogenes* Tü494. Phosphinothricin-acetyl-transferase (PAT) enzyme acetylates L-glufosinate-ammonium (Thompson et al., 1987; Wohlleben et al., 1988; Eckes et al., 1989)	Event Bt11 expresses the PAT protein which confers tolerance to glufosinate ammonium-based herbicides (Droge-Laser et al., 1994)
MIR162	Vip3Aa20	Based on a gene from *Bacillus thuringiensis* strain AB88 (Estruch et al., 1996). In addition to Cry proteins, *B. thuringiensis* also produces insecticidal proteins during its vegetative growth stage. These are referred to as vegetative insecticidal proteins (Vip) (Fang et al., 2007)	Event MIR162 expresses a modified version of the *B. thuringiensis* *vip3Aa1* gene, and encodes Vip3Aa20, a protein toxic to certain lepidopteran larvae feeding on maize

Event	Protein	Donor organism and biological function	Intended effects in GM plant
	PMI[(a)]	Based on a gene from *Escherichia coli*. The phosphomannose isomerase (PMI) enzyme catalyses the isomerisation of mannose-6-phosphate to fructose-6-phosphate and plays a role in the metabolism of mannose (Markovitz et al., 1967)	Event MIR162 expresses PMI, which is used as selectable marker. Mannose normally inhibits root growth, respiration and germination. Transformed cells expressing PMI are able to utilise mannose as a carbon source (Negrotto et al., 2000)
MIR604	mCry3A	Based on genes from *Bacillus thuringiensis* subsp. *tenebrionis*. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal proteins (Cry) (Schnepf et al., 1998; Ellis et al., 2002)	Event MIR604 expresses a modified version of the native Cry3A protein (Chen and Stacy, 2003). mCry3A is a protein toxic to certain coleopteran larvae feeding on maize
	PMI[(a)]	Based on a gene from *E. coli*. PMI (phosphomannose isomerase) catalyses the isomerisation of mannose-6-phosphate to fructose-6-phosphate and plays a role in the metabolism of mannose (Markovitz et al., 1967)	Event MIR604 expresses PMI, which is used as selectable marker. Mannose normally inhibits root growth, respiration and germination. Transformed cells expressing PMI are able to utilise mannose as a carbon source (Negrotto et al., 2000)
1507	Cry1F	Based on genes from *Bacillus thuringiensis* subsp. *aizawai*. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal protein (Cry) (Schnepf et al., 1998; Ellis et al., 2002)	Event 1507 expresses a truncated version of the Cry1F protein. Cry1F is a protein toxic to certain lepidopteran larvae feeding on maize
	PAT	Based on a gene from *Streptomyces viridochromogenes* Tü494. Phosphinothricin-acetyl-transferase (PAT) enzyme acetylates L-glufosinate-ammonium (Thompson et al., 1987; Wohlleben et al., 1988; Eckes et al., 1989)	Event 1507 expresses the PAT protein which confers tolerance to glufosinate ammonium-based herbicides (Droge-Laser et al., 1994)
5307	eCry3.1A	Based on genes from *Bacillus thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis*. *B. thuringiensis* is an insect pathogen; its insecticidal activity is attributed to the expression of crystal protein (Cry) (Schnepf et al., 1998; Ellis et al., 2002)	Event 5307 expresses the synthetic protein eCry3.1Ab which is a chimera composed of the N-terminal portion of the mCry3A and the C-terminal portion of the Cry1Ab protein. eCry3.1Ab is an insecticidal protein toxic to certain coleopteran larvae feeding on maize
	PMI[(a)]	Based on a gene from *E. coli*. Phosphomannose isomerase (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)	Event 5307 expresses PMI, which is used as selectable marker. Mannose normally inhibits root growth, respiration and germination. Transformed cells expressing PMI are able to utilise mannose as a carbon source (Negrotto et al., 2000)
GA21	mEPSPS	Based on a gene from *Zea mays*. 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Herrmann, 1995)	Event GA21 expresses mEPSPS protein which is a modified version of the endogenous EPSPS enzyme that confers tolerance to glyphosate-based herbicides (Lebrun et al., 2003)

(a): It includes the PMI expressed in MIR162 and 5307 and that expressed in MIR604. These two PMI variants differ by two amino acids.

3.3.1.2. Integrity of the events in maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21

The genetic stability of the inserted DNA over multiple generations in the single maize events Bt11, MIR162, MIR604, 1507, 5307 and GA21 was previously demonstrated (see Table 2). Integrity of these events in the six-event stack maize was demonstrated by Southern analyses.

3.3.1.3. Information on the expression of the inserts

Cry1Ab, PAT, Vip3Aa20, PMI, mCry3A, Cry1F, eCry3.1Ab and mEPSPS protein levels were analysed by enzyme-linked immunosorbent assay (ELISA) in material harvested from a field trial across three locations in the USA in 2012. Samples analysed included leaf (stage V6 and R1), root (V6 and R1), whole plant (V6 and R1), pollen (R1) and grain (R6 and senescence) not treated with intended herbicides.

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

The levels of the proteins newly expressed in the six-event stack and the corresponding singles were comparable in all tissues except for PAT and PMI, which showed the expected higher levels in the stack resulting from the combination of events Bt11 and 1507 (producing PAT) and the combination of events MIR162, 5307 and MIR604 (producing PMI) respectively (Appendix A). The mEPSPS expression levels measured for some of the analysed tissues were low (most samples were below the limit of quantification (LOQ)) and therefore quantitative data for mEPSPS in those tissues were derived from a limited number of samples. Based on the available data, there is no indication of interactions that may affect the levels of the newly expressed proteins in this stack.

3.3.1.4. Conclusions of the molecular characterisation

The molecular data establish that the events stacked in the six-event stack maize have retained their integrity. Protein expression analyses show that the levels of the newly expressed proteins are similar in the six-event stack maize and the single events except for the expected higher levels of PAT and PMI. Therefore, there is no indication of an interaction that may affect the levels of the newly expressed proteins in this stack.

Based on the known biological function of the newly expressed proteins, the only foreseen interactions at the biological level are between the Vip3Aa20 protein and the Cry proteins and between the Cry proteins in susceptible insects, which is dealt with in Section 3.3.4.4.

3.3.2. Comparative analysis[7]

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

Application EFSA-GMO-DE-2011-103 presents data on agronomic and phenotypic characteristics and on forage and grain composition of the six-event stack maize derived from field trials performed in the USA in 2012 (Table 5).

Table 5: Overview of comparative analysis studies with maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 provided in application EFSA-GMO-NL-2013-103

Study focus	Study details	Comparator	Commercial non-GM reference varieties
Agronomic and phenotypic characteristics and compositional analysis	Field trial study, 2012, USA, eight sites(a)	5XH751 × NP2222	Six(b)

(a): The field sites were located in: Atlantic, Richland and Bagley in Iowa; Seymour and Wyoming in Illinois; York in Nebraska; Delavan in Wisconsin and Larned in Kansas.
(b): H-6044, NK SYMBA, NK THERMO, X36344, H-7191 and H-7540. All six non-GM maize hybrids were grown at every location.

The field trials were conducted in major maize-growing areas of the USA, representing regions of diverse agronomic practices and environmental conditions. All the materials were treated with plant protection products (PPP) according to local requirements. At each site, the following materials were grown in a randomised complete block design with four replications: the six-event stack maize not

[7] Dossier: Part II – Section A.3; additional information: 4/5/2018 and 18/9/2018.

treated with the intended herbicides, the six-event stack maize treated with glyphosate- and glufosinate-ammonium-containing herbicides, a non-GM comparator and six non-GM maize reference varieties. The six-event stack maize was obtained by conventional crossing of the six single events. Events Bt11, MIR162, MIR604, 5307 and GA21 were introgressed via backcrossing in the inbred line NP2222, while event 1507 in 5XH751. As documented by the pedigree, the six single events, after backcrossing, were combined in a hybrid maize with genetic background (F_1) 5XH751 × NP2222. The same two inbred lines (5XH751 and NP2222) were crossed to produce the non-GM hybrid maize used as comparator (5XH751 × NP2222). The GMO Panel considered the selected non-GM comparator suitable.

The GMO Panel noted that the comparative relative maturity of the selected non-GM reference varieties was shorter than the optimal range for the chosen sites. However, the GMO Panel considered that at most of the field trial sites sowing occurred relatively late, close to the limit of the typical range. Therefore, considering the specific conditions under which the materials were grown and in light of the typical range of growing conditions in USA, the non-GM reference varieties are considered acceptable.

The seeds of the six-event stack hybrid maize and of its non-GM comparator that were sown to conduct the comparative assessment studies were produced under similar conditions. The seeds were also tested for their quality and germinability and were considered by the GMO Panel to be of adequate quality.

Statistical analysis of field trial data

The statistical analysis of the agronomic, phenotypic and compositional data from the field trials followed the recommendations of the GMO Panel (EFSA GMO Panel, 2010e, 2011a). This includes, for each of the two treatments of the six-event stack maize, the application of a difference test (between the GM maize and the non-GM comparator) and an equivalence test (between the GM maize and the set of non-GM commercial reference varieties). The results of the equivalence test are categorised into four possible outcomes (I–IV, ranging from equivalence to non-equivalence).[8]

3.3.2.2. Agronomic and phenotypic characterisation

Fourteen traits related to crop physiology, morphology, development and yield were measured.[9] Of those, five were not analysed as described in Section 3.3.2.1 because of high discreteness and lack of variability in the data.[10]

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

- For the six-event stack maize (not treated with the intended herbicides), statistically significant differences with the non-GM comparator were identified for early stand count, final stand count, days to 50% pollen shed and days to 50% silking. All these endpoints fell under equivalence category I or II, except for early and final stand count which fell under equivalence category IV.[11] Grain moisture fell under equivalence category IV; however, no significant differences were identified with the non-GM comparator.
- For the six-event stack maize (treated with the intended herbicides), statistically significant differences with the non-GM comparator were identified for early stand count, final stand count, days to 50% pollen shed, days to 50% silking, ear height and grain moisture. All these endpoints fell under equivalence category I or II, except for early stand count, final stand count, days to 50% pollen shed and grain moisture, which fell under equivalence category III or IV.[12]

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

[9] The traits were: early stand count, final stand count, early growth rating (seedling vigour), days to 50% pollen shed, days to 50% silking, ear height, plant height, stalk lodged plants, root lodged plants, stay green, dropped ears, grain yield, grain moisture and test weight.

[10] Early growth rating, stay green, root lodged plants, stalk lodged plants and dropped ears.

[11] Estimated means for early stand count: 62.1 (untreated GM maize), 59.1 (treated GM maize), 75.8 (non-GM comparator) and 76.5 (non-GM reference varieties); equivalence limits: (71.3, 81.7). Estimated means for final stand count: 60.8 (untreated GM maize), 58.0 (treated GM maize), 72.7 (non-GM comparator) and 72.8 (non-GM reference varieties); equivalence limits: (67.5, 78.1).

[12] Estimated means for days to 50% pollen shed: 57.8 (untreated GM maize), 58.1 (treated GM maize), 56.5 (non-GM comparator) and 54.1 (non-GM reference varieties); equivalence limits: (50.4, 57.8). Estimated means for grain moisture (%): 17.5 (untreated GM maize), 17.2 (treated GM maize), 18.1 (non-GM comparator) and 14.9 (non-GM reference varieties); equivalence limits: (13.1, 16.7).

For days to 50% pollen shed and grain moisture, the estimated mean values for the GM maize and the non-GM comparator are higher than those for the non-GM reference varieties. The GMO Panel considers that these results are linked to the relatively short cycle of the selected non-GM reference lines (see Section 3.3.2.1). Although it is not clear why early and final stand count were reduced, the GMO Panel considers that these differences do not affect the use of the field trial data for the comparative analysis, as no changes were observed for the other yield components (yield and test weight). Whether the differences identified can lead to an environmental adverse effect is considered in Section 3.3.4.

Additionally, no altered stress responses of the six-event stack maize were observed compared with its non-GM comparator with regard to visually observable responses to naturally occurring diseases, arthropod damage and abiotic stressors.

3.3.2.3. Compositional analysis

Maize forage and grains harvested from the field trial study in the USA in 2012 were analysed for 82 constituents (9 in forage and 73 in grain), including the key constituents recommended by OECD (OECD, 2002). The statistical analysis was not applied to 17 grain constituents[13] because more than half of the observations were below the limit of quantification. Moisture level was also not analysed, as the values reported do not refer to the moisture content of the grains at the time of harvesting but to the content following mechanical drying of the grains in the field before the laboratory analysis.

The statistical analysis was applied to the remaining 64 constituents (9 in forage[14] and 55 in grain[15]); a summary of the outcome of the test of difference and the test of equivalence is presented in Table 6.

- For the six-event stack maize (not treated with the intended herbicides), significant differences with the non-GM comparator were identified for 25 endpoints (2 in forage and 23 in grain); of those, six grain endpoints fell under equivalence category III/IV (ash, β-carotene, ferulic acid, potassium, zinc and methionine) while the other endpoints fell under category I/II. Six more endpoints fell under equivalence category III/IV (Table 6); for those, however, no significant differences with the non-GM comparator were identified.
- For the six-event stack maize (treated with the intended herbicides), significant differences with the non-GM comparator were identified for 24 endpoints (1 in forage and 23 in grain); of those, eight grain endpoints fell under equivalence category III/IV (ash, β-carotene, folic acid, ferulic acid, potassium, zinc, arachidic acid (C20:0) and methionine) while the other endpoints fell under category I/II. Four more endpoints fell under equivalence category III/IV (Table 6); for those, however, no significant differences with the non-GM comparator were identified.

[13] Caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), γ-linolenic acid (C18:3), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), selenium, sodium, furfural and raffinose.

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

[15] Proximates and fibre content (protein, fat, ash, carbohydrates, acid detergent fibre (ADF), neutral detergent fibre (NDF) and total detergent fibre (TDF)), starch, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc), vitamins (β-carotene, thiamine, riboflavin, niacin, pyridoxine, folic acid and α-tocopherol), amino acids (alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine), fatty acids (palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), eicosenoic acid (C20:1) and behenic acid (C22:0)) and other compounds (ferulic acid, inositol, *p*-coumaric acid, phytic acid and trypsin inhibitor).

Table 6: Outcome of the comparative compositional analysis of grains and forage from maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21. The table shows the number of endpoints in each category

		Test of difference[b]			
		Not treated[c]		Treated	
		Not different	Significantly different	Not different	Significantly different
Test of equivalence[b]	Category I/II	32	19[d]	35	16[d]
	Category III/IV	6[e]	6[f]	4[e]	8[f]
	Not categorised	1[g]	_[h]	1[g]	_[h]
	Total endpoints	64		64	

(a): Comparison between the six-event stack maize and the non-GM comparator.
(b): Four different outcomes: category I (indicating full equivalence to the non-GM reference varieties); category II (equivalence is more likely than non-equivalence); category III (non-equivalence is more likely than equivalence); and category IV (indicating non-equivalence). Not categorised means that the test of equivalence was not applied because of the lack of variation among the non-GM reference varieties.
(c): Treated with intended herbicides glyphosate and glufosinate-ammonium.
(d): Endpoints with significant differences between the six-event stack maize and its non-GM comparator and falling in equivalence category I/II. For grains, for both treated and non-treated GM: NDF, manganese, phosphorus, glycine, threonine, oleic acid (C18:1), linoleic acid (C18:2), phytic acid and pyridoxine. Only non-treated: fat, iron, arginine, cystine, palmitoleic acid (C16:1), linolenic acid (C18:3), inositol and riboflavin. Only treated: protein, ADF, glutamic acid, proline, serine and behenic acid (C22:0). For forage, for both treated and non-treated GM: moisture. Non-treated only: phosphorus.
(e): Endpoints with no significant differences between the six-event stack maize and its non-GM comparator and falling in equivalence category III/IV. For grains, for both treated and non-treated GM: TDF, stearic acid (18:0) and thiamine. Only non-treated: arachidic acid (C20:0) and folic acid. For forage, for both treated and non-treated GM: protein.
(f): Endpoints with significant differences between the six-event stack maize and its non-GM comparator and falling in equivalence category III/IV. Quantitative results are reported in Table 7.
(g): Endpoints not categorised for equivalence and without significant differences between the six-event stack maize and its non-GM comparator: trypsin inhibitor in grain (both treated and not treated).
(h): Endpoints not categorised for equivalence and with significant differences between the six-event stack maize and its non-GM comparator: none.

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

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

Endpoint	Maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21		Non-GM comparator 5XH751 × NP2222	Non-GM reference varieties	
	Non-treated	Treated[a]		Mean	Equivalence limits
Ash (% dw)	1.45*	1.47*	1.34	1.34	1.25–1.44
Potassium (mg/kg dw)	4,070*	4,100*	3,670	3,470	3,110–3,850
Zinc (mg/kg dw)	18.0*	18.3*	20.3[b]	24.7	21.2–29.0
β-Carotene (mg/100 g dw)	0.073*	0.076*	0.086	0.132	0.077–0.224
Folic acid (mg/100 g dw)	0.051	0.055*	0.049[b]	0.040	0.036–0.045
Methionine (mg/g dw)	1.99*	1.98*	2.10[b]	2.29	2.12–2.48
Arachidic acid (C20:0) (% FA)	0.48	0.48*	0.47	0.43	0.38–0.48
Ferulic acid (mg/kg dw)	2,970*	2,970*	2,750	2,230	1,790–2,780

dw: dry weight; FA: total fatty acid.
(a): Treated with the intended herbicides glyphosate and glufosinate-ammonium.
(b): For folic acid, zinc and methionine, the mean value of the non-GM comparator was out of the equivalence limits derived from the non-GM commercial reference varieties.

For the six-event stack maize, significantly different values are marked with an asterisk, while the outcomes of the test of equivalence are differentiated by greyscale backgrounds: white (equivalence category I or II), light grey (equivalence category III) and dark grey (equivalence category IV).
For all endpoints, the analysis was done on a logarithmic scale, and the results were transformed back to the original scale.

3.3.2.4. Conclusions of the comparative analysis

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

- None of the differences identified in the agronomic and phenotypic characteristics tested between the six-event stack maize and the non-GM comparator needs further assessment, except for the changes in early stand count, final stand count, days to 50% pollen shed and grain moisture. These differences are further assessed for their potential environmental impact in Section 3.3.4.
- None of the differences identified in forage and grain composition between the six-event stack maize and the non-GM comparator needs further food/feed safety assessment except for the changes in levels of ash, potassium, zinc, β-carotene, folic acid, methionine, arachidic acid and ferulic acid. These differences are further discussed in Section 3.3.3.

3.3.3. Food and feed safety assessment[16]

3.3.3.1. Effects of processing

The six-event stack maize will undergo existing production processes used for conventional maize. Considering the changes observed in the compositional comparative analysis (Section 3.3.2.3), the processing of the six-event stack maize into food and feed products is not expected to result in products being different from those from conventional non-GM maize varieties.

3.3.3.2. Influence of temperature and pH on newly expressed proteins

The effects of temperature and pH on the newly expressed proteins in this six-event stack maize have been previously evaluated by the GMO Panel (Table 2). No new information has been provided in the context of this application.

3.3.3.3. Toxicology

Testing of newly expressed proteins

Eight proteins (Cry1Ab, Vip3Aa20, mCry3A, Cry1F, eCry3.1Ab, PAT, PMI[17] and mEPSPS) are newly expressed in the six-event stack maize (Section 3.3.1). The GMO Panel has previously assessed these proteins in the context of the single maize events (Table 2) and no safety concerns were identified for humans and animals. The GMO Panel is not aware of any new information that would change these conclusions.

The potential for a functional interaction between the proteins newly expressed in this six-event stack maize has been assessed with regard to human and animal health. The insecticidal proteins Cry1Ab, mCry3A, Cry1F and eCry3.1Ab are delta-endotoxins acting through cellular receptors found in target insect species. It is reported that the gastrointestinal tract of mammals, including humans, lacks receptors with high-specific affinity to Cry proteins (Hammond et al., 2013; Koch et al., 2015). The Vip3Aa20 protein is a protein secreted by *B. thuringiensis* during its vegetative phase acting in target insects via a mechanism similar to that of Cry proteins (Bel et al., 2017; Chakroun et al., 2016). The PAT, PMI and mEPSPS proteins are enzymes that catalyse distinct biochemical reactions and act on unrelated substrates with high substrate specificity.

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

The expression levels of the newly expressed proteins in the six-event stack maize were similar to those of the single events with the exception of PMI and PAT (Section 3.3.1). Total levels of these newly expressed proteins were consistently higher in the tissues of the six-event stack maize than in

[16] Dossier: Part II – Sections A3.5, A4, A5 and A6.
[17] It includes the PMI expressed in MIR162 and 5307 and that expressed in MIR604. These two PMI variants differ by two amino acids (see Table 4).

those of the individual events. This could be expected, given the introduction of multiple copies of the respective genes. Introduction of PMI activity in the single events did not result in changes of relevant endogenous compounds (i.e. sugars, sugar alcohols and sugar phosphates) compared with the conventional counterparts (Table 2). There is no expectation that this enzyme would also have impact on carbohydrate metabolism in the six-event stack maize.

In vitro protein degradation studies on Cry1Ab, Vip3Aa20, mCry3A, Cry1F, eCry3.1Ab, PAT, PMI[17] and mEPSPS proteins have been previously evaluated by the GMO Panel (Table 2). In the context of this application, no new studies addressing *in vitro* protein degradation of these newly expressed proteins were provided by the applicant.

The GMO Panel concludes that there are no safety concerns to human and animal health related to the newly expressed proteins Cry1Ab, Vip3Aa20, mCry3A, Cry1F, eCry3.1Ab, PAT, PMI and mEPSPS in maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21.

Testing of new constituents other than proteins

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

Information on altered levels of food and feed constituents

The levels of ash, potassium, zinc, β-carotene, folic acid, methionine, arachidic acid (C20:0) and ferulic acid in grains from the six-event stack maize were significantly different from its non-GM comparator and showed a lack of equivalence with the set of non-GM commercial reference varieties (Section 3.3.2.3). Taking into account the biological characteristics and functions of these compounds, the observed differences are considered of no toxicological concern by the GMO Panel. Further information on the safety of these maize constituents is provided in Section 3.3.3.5.

Testing of the whole genetically modified food and feed

No animal studies with the food/feed derived from the six-event stack maize were provided. Based on the outcome of the studies considered in the molecular characterisation and comparative analysis, no substantial modifications of toxicological concern in the composition of the six-event stack maize, and no indication of possible unintended effects relevant to food/feed safety have been identified (see Sections 3.3.1 and 3.3.2.3). Therefore, animal studies on food/feed derived from the six-event stack maize are not necessary (EFSA GMO Panel, 2011a).

3.3.3.4. Allergenicity

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

Assessment of allergenicity of the newly expressed proteins

For allergenicity, the GMO Panel has previously evaluated the safety of Cry1Ab, PAT, Vip3Aa20, PMI,[17] mCry3A, Cry1F, eCry3.1Ab and mEPSPS proteins individually, and no concerns on allergenicity were identified in the context of the applications assessed (see Table 2). No new information on allergenicity of these proteins that might change the previous conclusions of the GMO Panel has become available. Based on the current knowledge, and as none of the newly expressed proteins showed allergenicity, no reasons for concerns regarding the simultaneous presence of these newly expressed proteins in the six-event stack maize affecting their allergenicity are expected.

For adjuvanticity, the Bt protein Cry1Ac has been suggested to possess adjuvant activity based on animal studies when applied at relatively high doses (e.g. Vázquez et al., 1999). The Panel has previously evaluated the safety of Cry1Ab, Vip3Aa20, mCry3A, Cry1F and eCry3.1Ab proteins, and no concerns on adjuvanticity were identified in the context of the applications assessed (see Table 2). The levels of the individual Bt proteins in the six-event stack maize are similar to those in the respective single maize events (see Section 3.3.1.3). From the limited evidence available, the GMO Panel does

not find indications that the presence of the Bt proteins at the levels expressed in the six-event stack maize might act as adjuvants with the potential to enhance a specific immunoglobulin E (IgE) response and to favour the development of an allergic reaction.

Assessment of allergenicity of GM plant products

The GMO Panel regularly reviews the available publications on food allergy to maize. However, maize is not considered a common allergenic food[18] (OECD, 2002). Therefore, the GMO Panel does not request experimental data to analyse the allergen repertoire of GM maize.

The applicant provided spontaneous information where levels of lipid transfer protein (LTP, a known allergen also present in maize) in the six-event stack maize were compared to those in a non-GM comparator and commercial reference varieties. No changes in expression levels raising concern were identified.

In the context of this application and considering the data from the molecular characterisation, the compositional analysis and the assessment of the newly expressed proteins (see Sections 3.3.1, 3.3.2 and 3.3.3.3), the GMO Panel identifies no indications of a potentially increased allergenicity of food and feed derived from the six-event stack maize with respect to those derived from its non-GM comparator.

3.3.3.5. Nutritional assessment of GM food/feed

The intended trait of the six-event stack maize is insect resistance and herbicide tolerance, with no intention to alter the nutritional parameters. However, the levels of ash, potassium, zinc, β-carotene, folic acid, methionine, arachidic acid (C20:0) and ferulic acid in grains from the six-event stack maize were significantly different from the non-GM comparator and showed a lack of equivalence with the set of non-GM commercial reference varieties (Section 3.3.2.3). The biological roles of these compounds, the contribution of maize to their total intake and the magnitude and direction of the observed changes are considered in the nutritional assessment.

Human nutrition

Statistically significant differences were identified for potassium and zinc. Regarding potassium, no tolerable upper intake level (UL) exists, therefore the observed increase of around 10% is considered irrelevant from a nutritional point of view. Zinc is an essential mineral in the diet, being an important part of the structure of certain proteins and serving as a cofactor for many enzymes. The main dietary sources of zinc are foods of animal origin, particularly meat, milk and their derived products (EFSA NDA Panel, 2014). Although cereals are also considered relevant contributors to the total intake of zinc in the diet, it is widely recognised that mineral bioavailability is substantially decreased due to the presence of phytic acid (Suri and Tanumihardjo, 2016). The quantity of dietary zinc available for absorption strongly depends on overall dietary levels of phytate intake (EFSA NDA Panel, 2014). Therefore, the decrease of approximately 10% of zinc levels is not considered relevant. The increased levels of ash in the GM-maize are probably explained by the higher levels of salts of potassium, the most abundant mineral in maize.

β-Carotene is a precursor of vitamin A and is found in plant derived foods; together with preformed vitamin A (mainly retinol and retinyl esters) present in foods of animal origin it contributes to the total dietary intake of vitamin A. Milk, meat, vegetables and derived products are the main sources of vitamin A in the diet. Cereals and cereal-based products contribute much less to the total intake of vitamin A, with contributions in the adult population ranging between 2.7% and 6.5% of the total (average contribution = 4.1%), with the maximum contribution (10%) estimated in adolescents (EFSA NDA Panel, 2015). The decrease observed in β-carotene is not considered relevant from a nutritional point of view. An increase of up to 12% of folic acid (folate, the natural form present in food) was observed in the six-event stack maize as compared to the non-GM comparator. Green vegetables and certain (citrus) fruits are important dietary sources of folates. Although UL are set for folic acid, the relatively high upper limits (200–1,000 μg/day[19]) as compared to the levels present in maize (~ 0.005 mg/100 g dw) make the observed changes irrelevant from a nutritional point of view.

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

[19] Tolerable upper intake levels (UL) refer to folic acid, the synthetic form typically used as supplement and with higher bioavailability than food folate (0.5 μg of a folic acid supplement taken on an empty stomach = 1 μg food folate).

Methionine is an essential amino acid containing sulfur; maize possesses an amino acid pattern that covers 132% of the sulfur amino acid (SAA) pattern requirements; therefore, the nutritional impact of the observed decrease (~6%) is considered negligible. An increase of arachidic acid (C20:0) levels was observed in the six-event stack maize; however, considering the minor relative amount of this fatty acid in maize (less than 0.5% of total FA) and the negligible contribution of maize to its dietary intake as compared to other dietary sources (e.g. peanut oil), the slight increase is not nutritionally relevant. Ferulic acid (4-hydroxy-3-methoxycinnamic) is the most abundant phenolic compound in maize grain (Boz, 2015; Bento-Silva et al., 2018). Maize is one of the most important dietary sources of ferulic acid in humans. No toxicity has been linked to the dietary intake of ferulic acid, being a phenolic compound easily absorbed and metabolised in humans. Therefore, the observed increase in the six-event stack maize is not considered nutritionally relevant.

Animal nutrition

Ash is a component of the proximates and provides a measure of the total amount of minerals present in feed, without offering a qualitative and quantitative insight for specific minerals. Moreover, animal requirements for ash are not provided. Therefore, the nutritional impact of the increase (~8%) is considered negligible. Potassium and zinc are essential minerals naturally occurring in feed material (e.g. plant and plant products), of importance for their physiological function in the metabolism of animals. Animal diets are usually balanced with mineral supplements according to the foreseen uses, therefore the observed changes are not considered nutritionally relevant and do not pose a risk for animals. Considering that the significance of β-carotene in diets for animals is negligible because these are supplemented with vitamin A, the observed lower level would not affect its total dietary intake in the European animal population. Folic acid, rarely analysed in animal feed, is widely distributed in nature (e.g. green leafy materials, cereals) and it is synthesised by microbes in the rumen. Underfeeding of this acid should be avoided because it has an important role in the metabolism, especially during pregnancy, for RNA and DNA synthesis of fetal and placental tissues. The direction and magnitude of the change observed do not pose a concern for animal nutrition. Methionine is an essential amino acid for all animals; however, the nutritional impact of the observed decrease (~6%) can be considered negligible, and diets are also balanced with synthetic amino acids, as needed. Arachidic acid is a saturated fatty acid structurally related to arachidonic acid. The magnitude of the increase of arachidic acid content is considered negligible with no nutritional impact for animals, also considering the minor amount of this fatty acid in maize. Ferulic acid is not considered a major element in animal nutrition. Among whole grain cereals, maize has the highest content in ferulic acid, but other sources of ferulic acid in animal nutrition are normally used, such as grain brans and sugar beet pulp. The magnitude of the change observed does not pose a concern for animal nutrition.

Conclusion on human and animal nutrition

Based on the current knowledge of the biological role of the compounds assessed, the magnitude and direction of the changes identified, and the relevance of maize as contributor to the intake of these compounds, the GMO Panel concludes that the nutritional impact of foods and feeds from the six-event stack maize is expected to be the same as that of foods and feeds derived from the comparator and non-GM reference varieties.

3.3.3.6. Conclusion of the food and feed safety assessment

The proteins Cry1Ab, PAT, Vip3Aa20, PMI, mCry3A, Cry1F, eCry3.1Ab and mEPSPS newly expressed in the six-event stack maize do not raise safety concerns for human and animal health. Interactions between these newly expressed proteins raising food and feed safety concerns (toxicological, allergenicity and adjuvanticity) are not expected. The nutritional impact of the six-event stack maize foods and feeds is expected to be the same as those from the comparator and non-GM reference varieties. The GMO Panel concludes that the six-event stack maize, as described in this application, is as safe as and nutritionally equivalent to the comparator and the non-GM reference varieties tested.

3.3.4. Environmental risk assessment[20]

Considering the scope of application EFSA-GMO-DE-2011-103, which excludes cultivation, the environmental risk assessment (ERA) of maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21

[20] Dossier: Part II – Section E; additional information: 8/2/2019.

mainly takes into account: (1) the exposure of microorganisms to recombinant DNA in the gastrointestinal tract of animals fed GM material and of microorganisms present in environments exposed to faecal material of these animals (manure and faeces); and (2) the accidental release into the environment of viable six-event stack maize grains during transportation and/or processing (EFSA GMO Panel, 2010a).

3.3.4.1. Persistence and invasiveness of the GM plant

Maize is highly domesticated, not winter hardy in colder regions of Europe, and generally unable to survive in the environment without appropriate management. Occasional feral GM maize plants may occur outside cultivation areas in the EU (e.g. Pascher, 2016), but survival is limited mainly by a combination of low competitiveness, absence of a dormancy phase and susceptibility to plant pathogens, herbivores and cold climate conditions (OECD, 2002). Field observations indicate that maize grains may survive and overwinter in some EU regions, resulting in volunteers in subsequent crops (e.g. Gruber et al., 2008; Palaudelmás et al., 2009; Pascher, 2016). However, maize volunteers have been shown to grow weakly and flower asynchronously with the maize crop (Palaudelmás et al., 2009). Thus, the establishment and survival of feral and volunteer maize in the EU is currently limited and transient.

It is unlikely that the intended traits of the six-event stack maize and the observed decreased in early stand count, final stand count, days to 50% pollen shed and grain moisture (see Section 3.3.2.2) will provide a selective advantage to maize plants, except when they are exposed to glyphosate- and/or glufosinate-ammonium-containing herbicides or infested by insect pests that are susceptible to the *Bt* proteins expressed by this GM maize. However, this fitness advantage will not allow the six-event stack maize to overcome other biological and abiotic factors (described above) limiting plant's persistence and invasiveness. Therefore, the presence of the intended traits and the observed differences in early stand count, final stand count, days to 50% pollen shed and grain moisture will not affect the persistence and invasiveness of the GM plant.

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

3.3.4.2. Potential for gene transfer

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

Plant-to-microorganism gene transfer

The probability and potential adverse effects of HGT of the recombinant DNA have been assessed in previous GMO Panel Scientific Opinions for the single events (see Table 2). No concern as a result of an unlikely, but theoretically possible, horizontal gene transfer of the recombinant genes to bacteria in the gut of domesticated animals and humans fed GM material or other receiving environments was identified. The applicant submitted an updated bioinformatic analysis for each of the single events in order to assess the possibility for HGT by homologous recombination.

The updated bioinformatics analysis for maize event Bt11 revealed elements that are intervening sequences flanking the cry1Ab expression cassette. These could provide sufficient length and sequence identity to plasmid bacterial DNA, as typically present in bacterial cloning vectors, to allow double homologous recombination. The analysis revealed a number of possible recipient organisms including *E. coli*; however, the size of the non-homologous insert is relatively extensive (> 3100 bp), which decreases recombination efficiency (EFSA, 2017b). The cry1Ab is of bacterial origin and it is plant codon-optimised and under the control of plant virus elements and thus unlikely to be functional in bacteria. The analysis also identified a ~ 250 bp-long DNA sequence of sufficient identity to a single site on an *A. tumefaciens* nopaline Ti plasmid. This would not increase the potential for facilitated HGT by double homologous recombination.

The bioinformatic analyses of maize events MIR162, MIR604 and 5307 revealed similar results. In all three events, the *pmi* gene derived from *E. coli* and the T-nos terminator of an *A. tumefaciens* nopaline plasmid were identified as elements with sufficient length and sequence identity to facilitate homologous recombination. Considering that these sequences do not occur in the same bacterial species, there is no indication for facilitated double homologous recombination for these three events.

The bioinformatic analysis of maize event GA21 identified a ~250 bp-long DNA sequence, present in three copies in the event, of sufficient identity to a single site on an *A. tumefaciens* nopaline Ti plasmid. Because of the lack of a second site with sequence identity, no increased potential for facilitated HGT by double homologous recombination was identified.

The updated bioinformatic analysis provided in this application for maize event 1507 confirms the assessments provided in the context of previous applications (EFSA GMO Panel, 2019a,b).

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

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

Plant-to plant-gene transfer

The GMO Panel assessed the potential for occasional feral six-event stack maize plants originating from grain import spills to transfer recombinant DNA to sexually compatible plants; the environmental consequences of this transfer were also considered.

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

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

The potential of spilled maize grains to establish, grow and produce pollen is extremely low and transient (see Section 3.3.4.1). Therefore, likelihood/frequency of cross-pollination between occasional feral GM maize plants resulting from grain spillage, and weedy or cultivated *Zea* plants is considered extremely low (EFSA, 2016). Even if cross-pollination would occur, the GMO Panel is of the opinion that environmental effects as a consequence of the spread of genes from occasional feral GM maize plants in Europe will not differ from that of conventional maize varieties for the reasons given in Section 3.3.4.1, even if exposed to the intended herbicides.

3.3.4.3. Interactions of the GM plant with target organisms

Taking the scope of application EFSA-GMO-DE-2011-103 into account (no cultivation), potential interactions of occasional feral six-event stack maize plants arising from grain import spills with target organisms are not considered a relevant issue by the GMO Panel.

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

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

3.3.4.5. Interactions with the abiotic environment and biogeochemical cycles

Given that environmental exposure to spilled grains or occasional feral six-event stack maize plants arising from grain import spills is limited, and because most proteins are degraded before entering the environment through faecal material of animals fed GM maize, potential interactions with the abiotic environment and biogeochemical cycles are not considered to raise any environmental safety concern.

3.3.4.6. Conclusion of the environmental risk assessment

The GMO Panel concludes that it is unlikely that the six-event stack maize would differ from conventional maize varieties in its ability to persist under EU environmental conditions. Considering the scope of application EFSA-GMO-DE-2011-103, interactions of occasional feral six-event stack maize plants with the biotic and abiotic environment are not considered to be relevant issues. The analysis of

HGT from the six-event stack maize to bacteria does not indicate a safety concern. Therefore, considering the combined traits, the outcome of the comparative analysis, and the routes and levels of exposure, the GMO Panel concludes that the six-event stack maize would not raise safety concerns in the event of accidental release of viable GM maize grains into the environment.

3.3.5. Conclusion on maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21

No new data on the single maize events Bt11, MIR162, MIR604, 1507, 5307 and GA21 leading to a modification of the original conclusions on their safety were identified.

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

Considering the combined traits and their interactions, the outcome of the comparative analysis, and routes and levels of exposure, the GMO Panel concludes that the six-event stack maize would not raise safety concerns in the event of accidental release of viable GM maize grains into the environment.

No scientific information that could change the conclusions on this six-event stack was retrieved in a literature search covering the period since the time of validity of the application.[21]

In conclusion, the GMO Panel considers that the six-event stack maize is as safe as its non-GM comparator and the tested non-GM maize reference varieties with respect to potential effects on human and animal health and the environment.

3.4. Risk assessment of the subcombinations[22]

Subcombinations previously assessed in the frame of other applications are discussed in Section 3.4.1.

The strategy followed for the assessment of those subcombinations which have not been previously assessed has been described by the GMO Panel.[23] In this case, the risk assessment takes as its starting point the assessment of the single maize events, and uses the data generated for the six-event stack, as well as all the additional data available on subcombinations previously assessed by the GMO Panel (Table 2) and the new information on subcombinations not yet assessed.

3.4.1. Subcombinations previously assessed

The GMO Panel has previously assessed 22 subcombinations (10 two-event stacks, 9 three-event stacks and 3 four-event stacks; see Table 2) and did not identify any safety concern. No new scientific information relevant to the risk assessment of these maize stacks became available since the validation of application EFSA-GMO-DE-2011-103. Consequently, the GMO Panel considers that its previous conclusions on these subcombinations remain valid.

3.4.2. Subcombinations not previously assessed

Of the 56 subcombinations included in the scope of this application, 34 have not been assessed previously by the GMO Panel (see Table 8).

[21] Additional information: 30/8/2018.
[22] Additional information: 29/5/2018.
[23] 115th GMO Panel meeting (Annex 1 of the minutes: http://www.efsa.europa.eu/sites/default/files/event/170517-m.pdf).

Table 8: Subcombinations of maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 not previously assessed and covered by the scope of application EFSA-GMO-DE-2011-103

Degree of Stacking	Events
Five-event stack maize	Bt11 × MIR162 × MIR604 × 1507 × 5307
	Bt11 × MIR162 × MIR604 × 1507 × GA21
	Bt11 × MIR162 × MIR604 × 5307 × GA21
	Bt11 × MIR162 × 1507 × 5307 × GA21
	Bt11 × MIR604 × 1507 × 5307 × GA21[(a)]
	MIR162 × MIR604 × 1507 × 5307 × GA21
Four-event stack maize	Bt11 × MIR162 × MIR604 × 1507
	Bt11 × MIR162 × MIR604 × 5307
	Bt11 × MIR604 × 1507 × 5307[(a)]
	Bt11 × MIR162 × 1507 × 5307
	Bt11 × MIR162 × 5307 × GA21
	Bt11 × MIR604 × 5307 × GA21
	Bt11 × 1507 × 5307 × GA21
	MIR162 × MIR604 × 1507 × 5307
	MIR162 × MIR604 × 1507 × GA21
	MIR162 × MIR604 × 5307 × GA21
	MIR162 × 1507 × 5307 × GA21
	MIR604 × 1507 × 5307 × GA21
Three-event stack maize	Bt11 × MIR162 × 5307
	Bt11 × MIR604 × 5307
	Bt11 × 1507 × 5307
	Bt11 × 5307 × GA21
	MIR162 × MIR604 × 1507
	MIR162 × MIR604 × 5307
	MIR162 × 1507 × 5307
	MIR162 × 5307 × GA21
	MIR604 × 1507 × 5307[(a)]
	MIR604 × 5307 × GA21
	1507 × 5307 × GA21
Two-event stack maize	Bt11 × 5307
	MIR162 × 5307
	MIR604 × 5307
	1507 × 5307
	5307 × GA21

(a): Additional information was provided for this subcombination.

New information on three of these maize stacks became available since the validation of application EFSA-GMO-DE-2011-103. The information is discussed in Sections 3.4.2.1–3.4.2.3.

3.4.2.1. Additional information on maize MIR604 × 5307 × 1507

Expression of the events

The applicant provided a study, not previously assessed by the GMO Panel, on protein expression levels of the three-event stack MIR604 × 5307 × 1507 and the corresponding single events. The data provided in this study are considered of limited value since they were derived from plants grown in one location during one season. Therefore, these data are not representative and not in line with the applicable GMO Panel guidelines (EFSA GMO Panel, 2011a). Hence, the GMO Panel has not taken these data into account for the risk assessment.

Non-target organism risk assessment

The applicant provided a study assessing the risk of maize MIR604 × 5307 × 1507 to non-target organisms. The GMO Panel considers this study not relevant because potential interactions of this three-stacked event maize with non-target organisms are not relevant in the context of the scope of application EFSA-GMO-DE-2011-103, which excludes cultivation.

3.4.2.2. Additional information on maize Bt11 × MIR604 × 5307 × 1507

Insect resistance management plan

The applicant provided an insect resistance management plan for maize Bt11 × MIR604 × 5307 × 1507 in Canada. The GMO Panel considers that this report does not contain any experimental data and, therefore, it cannot be used to support the risk assessment of maize Bt11 × MIR604 × 5307 × 1507.

3.4.2.3. Additional information on maize Bt11 × MIR604 × 1507 × 5307 × GA21

Integrity of the events

Two studies on the integrity of the events in the five-event stack maize Bt11 × MIR604 × 1507 × 5307 × GA21, not previously assessed by the GMO Panel, were submitted. The data demonstrated integrity of these events in the five-event stack maize by Southern analyses.

Expression of the events

The applicant provided two studies, not previously assessed by the GMO Panel, on protein expression levels of the five-event stack maize Bt11 × MIR604 × 1507 × 5307 × GA21 and the corresponding single events. The data provided in one of these studies is considered of limited value since derived from plants grown in one location during one season. Therefore, these data are not representative and not in line with the applicable GMO Panel guidelines (EFSA GMO Panel, 2011a). Hence, the GMO Panel has not taken these data into account for the risk assessment.

The other study contains data derived from plants harvested from a field trial at three locations in Argentina in the 2012–2013 growing season and is therefore in line with the applicable GMO Panel guidelines. Cry1Ab, PAT, mCry3A, PMI, Cry1F, eCry3.1Ab and mEPSPS protein levels in this study were analysed by ELISA. Samples analysed included leaf (V6 and R1), root (V6 and R1), whole plant (V6 and R1), pollen (R1) and grain (R6 and senescence) not treated with intended herbicides. The levels of all the newly expressed proteins in the five-event stack and the corresponding singles are comparable in all examined tissues except for PAT and PMI which showed the expected higher levels in the stack resulting from the combination of events Bt11 and 1507 (producing PAT) and the combination of events MIR604 and 5307 (producing PMI) respectively. Therefore, the results are as expected (see Section 3.3.1.3).

Compositional analysis

The applicant provided a study on the compositional analysis of grain and forage of maize Bt11 × MIR604 × 1507 × 5307 × GA21. The data were obtained from field trials performed at eight locations in the USA in 2009. At each location, three materials were grown: the five-event stack maize and a non-GM comparator, both treated with maintenance pesticides, and the five-event stack maize treated with the intended herbicides. None of the differences in levels of nutritional compounds between the five-event stack maize and the non-GM comparator was found to raise food/feed safety issues. The GMO Panel notes that non-GM reference varieties were not included in the field trials and thus, the design of the field trials does not comply with the EFSA guidance applicable for application EFSA-GMO-DE-2011-103 (EFSA GMO Panel, 2011a). Therefore, the GMO Panel did not take the study into account for the risk assessment.

Agronomic and phenotypic characteristics

The applicant provided a study on the agronomic and phenotypic characteristics of maize Bt11 × MIR604 × 1507 × 5307 × GA21. The data were collected from a field trial study performed at eight locations in the USA in 2009. At each location, two materials were grown in a randomised complete block design with four replicates: the five-event stack maize and a non-GM comparator, both treated with maintenance pesticides. None of the differences in agronomic and phenotypic characteristics between the five-event stack maize and the non-GM comparator was found to raise

safety issues. The GMO Panel notes that the five-event stack maize treated with the intended herbicides and non-GM reference varieties were not included in the field trials and thus, the design of the field trial does not comply with the EFSA guidance applicable for application EFSA-GMO-DE-2011-103 (EFSA GMO Panel, 2011a). Therefore, the GMO Panel did not take the study into account for the risk assessment.

90-day feeding study in rats

The applicant provided a 90-day study in which pair-housed RccHan:WIST rats (10/sex per group) were randomly allocated to four groups and fed balanced diets containing approximately 10% or 41.5% (weight/weight) milled grain from maize Bt11 × MIR604 × 1507 × 5307 × GA21[24] and the respective same amount of a comparator. The study was conducted in accordance with OECD (1998) and in compliance with the OECD Principles of Good Laboratory Practice (GLP). The GMO Panel notes that the test material was derived from maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 by segregating out event MIR162. No information was provided on the treatment of GM maize with the intended herbicides. Moreover, no information was provided on the analytical composition of the GM and control maize or on the homogeneity of the test diets.

The statistical analysis (two-sided one-way analysis of variance (ANOVA) and, for organ weights, analysis of covariance using terminal kill body weight as covariate; all tests at 5% significance) compared each of the two test groups with the respective control. The individual animal was considered the experimental unit, except for feed consumption and related parameters (feed utilisation). No intercurrent deaths occurred and no test diet-related clinical signs were seen. The sporadic statistically significant differences observed between the test groups and their controls in body weight, body weight gain and feed consumption are considered not to be treatment-related. Overall feed utilisation (weeks 1–13) was decreased to a limited extent (~8%) in males given the 10% test diet as compared to their controls. This reduction was not associated with changes in the terminal body weight; therefore, it is considered not to be adverse. The isolated findings in a few functional observational battery parameters[25] and the minimally increased body temperature in females given the 41.5% test diet as compared to their controls (38.6 vs 38.0°C) are considered not to be adverse. Regarding clinical pathology, the statistically significant differences[26] observed between the rats given the test diets and their controls were minimal, not associated with changes in related endpoints and therefore are considered not to be adverse. Organ weight changes[27] observed in rats given the 41.5% test diet are considered not adverse based on the fact that no histopathological changes other than common background findings were observed in the affected organs in these groups. No gross pathology findings were noted related to the administration of the test diets; no test diet related differences were noted in incidence and severity of the histopathological findings between the groups.

Based on the results and the considerations above, the GMO Panel concludes that in this study no adverse effects were observed in rats given diets including up to 41.5% maize Bt11 × MIR604 × 1507 × 5307 × GA21 for 90 days.

42-day broiler study

A total of 540 (270 per sex) one-day-old chicken broilers (Ross 344 male × Ross 708 female) were randomly allocated to three dietary groups with 180 chicks per treatment (12 pens/treatment, half for each sex, 15 birds/pen) and fed balanced diets[28] containing up to 64% maize Bt11 × MIR604 × 1507 × 5307 × GA21 grain (test item) or from the comparator (control diet) or the commercial non-GM maize reference variety NCSU 2009 (reference diet). Diets (as crumbled pellets or pellets) and water were offered *ad libitum*.

[24] Identity confirmed by ELISA.

[25] Lower foregrip strength in females given the 10% test diet; higher motor activity measurements at the 6- to 10-minute time point in males given 10% test diet; and lower number of movements in males given the 41.5% test diet at the 26- to 30- and 31- to 35-minute intervals.

[26] 4% higher albumin in males and 1% higher sodium in females given the 10% test diet; 4% higher calcium in males and females given 41.5% test diet; 29% higher glucose, 27% lower AST, 21% lower ALP, 11% lower creatinine, 7% lower urea, 2% lower chloride and 1% lower sodium in males given the 41.5% test diet; 8% higher albumin and 6% higher total proteins in females given the 41.5% test diet.

[27] Higher mean absolute and relative testis weights and lower mean absolute and relative ovary and uterus weights (around 14% and 29%, respectively). It is noted that the mean weight of testes of the control at 41.5% is lower than that of the control group at 10% and the mean weight of uterus in controls at 41.5% is higher than that of the control group at 10%.

[28] Starter (0–15); grower (16–34); finisher (35–49).

The data were analysed with one- or two-way ANOVA (main factors: sex and diet). No statistically significant differences between the group fed test and control diets were observed in overall mortality (less than 2%), body weights, feed consumption and feed conversion rates feed conversion ratio among broilers fed the test, control and reference diets. Moreover, no statistically significant differences were observed in the carcass portions absolute and relative weights. However, the GMO Panel considered that a repeated measurement model should have been used for the analysis, including time and time-by-treatment interaction as additional fixed effects.

The GMO Panel notes that this study was not conducted according to GLP standards, and information regarding the treatment of test material with appropriate intended herbicides is missing; moreover, it is noted that the test material was derived from maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 by segregating out event MIR162.

Based on the results and the above considerations, the GMO Panel concludes that in this study no adverse effects were observed in boilers fed diets containing up to 64% maize Bt11 × MIR604 × 1507 × 5307 × GA21 grain. Moreover, the measured performance endpoints were similar between groups of animals fed balanced diets containing maize Bt11 × MIR604 × 1507 × 5307 × GA21 and the controls.

Endogenous LTP allergen assessment

The applicant provided spontaneous information where LTP levels in maize Bt11 × MIR604 × 1507 × 5307 × GA21 were compared to those in a non-GM comparator and commercial reference varieties. No changes in expression levels raising concern were identified.

3.4.2.4. Stability of the events

The genetic stability of the inserted DNA over multiple generations in the single maize events has been previously demonstrated (see Table 2). Integrity of the events was demonstrated in maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 (Section 3.3.1.2), the previously assessed maize subcombinations (Table 2) and also in the newly assessed data submitted for the five-event stack maize Bt11 × MIR604 × 1507 × 5307 × GA21 (Section 3.4.2.3). The GMO Panel finds no reasons to expect the loss of integrity of the events in the maize subcombinations not previously assessed.

3.4.2.5. Expression of the events

The GMO Panel assessed whether the combination of any of the six events by conventional crossing could result in significant changes in expression levels of the newly expressed proteins, as this could indicate an interaction between the events. Based on current knowledge of the molecular elements introduced, there is no reason to expect interactions that would affect the levels of the newly expressed proteins in the subcombinations compared with those in the single maize events. This assumption was confirmed by comparing the levels of the newly expressed proteins of each single maize event with those of the six-event stack maize and of a subcombination (five-event stack maize, Section 3.4.2.3). The levels in both stacks were comparable to the respective single events except for the expected higher levels of PAT and PMI. Therefore, there is no indication of an interaction manifesting at protein expression level. This supports the conclusion that interactions affecting expression levels of the newly expressed proteins are not expected in the maize subcombinations not previously assessed.

3.4.2.6. Potential interactions between the events

The GMO Panel assessed the potential for interactions between maize events in the 34 subcombinations not previously assessed (Table 8), taking into consideration the intended traits and unintended effects. Based on the known biological functions of the individual newly expressed proteins (Table 4), there is currently no expectation for possible interactions relevant for the food/feed or environmental safety between these proteins in those subcombinations. The GMO Panel also took into account all the intended and potential unintended effects considered in the assessment of the six single events, the previously assessed subcombinations (Table 2), the newly assessed five-event subcombination (Section 3.4.2.3) and the six-event stack maize. It is concluded that none of these events would raise safety concerns when combined in any of these maize subcombinations. The GMO Panel considers that no further data are needed to complete the assessment of subcombinations from the six-event stack maize.

3.4.3. Conclusion

Since no new safety concerns were identified for the previously assessed 22 subcombinations, the GMO Panel considers that its previous conclusions on these maize subcombinations remain valid. The remaining 34 subcombinations included in the scope of application EFSA-GMO-DE-2011-103 have not been previously assessed; for three of these, the applicant provided new information which was considered by the GMO Panel. For the 34 subcombinations, the GMO Panel assessed the possibility of interactions between the events and concludes that these combinations would not raise safety concerns. These subcombinations are therefore expected to be as safe as and nutritionally equivalent to the single maize events, the previously assessed subcombinations and the six-event stack maize.

3.5. Post-market monitoring[29]

3.5.1. Post-market monitoring of GM food/feed

The GMO Panel concludes that six-event stack maize, as described in this application, is nutritionally equivalent to and as safe as the non-GM comparator and the non-GM reference varieties tested (Section 3.3.5). Twenty-two of the subcombinations have been previously assessed and no safety concerns were identified. The 34 subcombinations not previously assessed and included in the scope of application EFSA-GMO-DE-2011-103 are expected to be as safe as and nutritionally equivalent to the single maize events, the previously assessed maize subcombinations and the six-event stack maize (Section 3.4.3). Therefore, the GMO Panel considers that post-market monitoring of food and feed from the six-event stack maize and its subcombinations, as described in this application, is not necessary.

3.5.2. Post-market environmental monitoring

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

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

As the ERA does not identify potential adverse environmental effects from the six-event stack maize, no case-specific monitoring is required.

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

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

3.5.3. Conclusion on post-market monitoring

No post-market monitoring of food and feed is necessary. The scope of the PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 and its subcombinations.

[29] Dossier: Part II – Sections D and E4.

4. Overall conclusions and recommendations

No new information on the six single maize events Bt11, MIR162, MIR604, 1507, 5307 and GA21 leading to a modification of the original conclusions on their safety were identified.

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

The GMO Panel concludes that there is a very low likelihood of environmental effects resulting from the accidental release of viable grains from the six-event stack maize into the environment.

Since no new data on the 22 subcombinations previously assessed (10 two-event stacks, 9 three-event stacks and 3 four-event stacks) that would lead to a modification of the original conclusions on their safety were identified, the GMO Panel considers that its previous conclusions on these maize stacks remain valid.

The remaining 34 subcombinations included in the scope of application EFSA-GMO-DE-2011-103 have not been previously assessed; for three of them, the applicant provided new information that was considered by the GMO Panel. The GMO Panel assessed possible interactions between the events in the 34 subcombinations, and concludes that these combinations of events Bt11, MIR162, MIR604, 1507, 5307 and GA21 would not raise safety concerns. These subcombinations are therefore expected to be as safe as and nutritionally equivalent to the maize single events, the previously assessed subcombinations and the six-event stack maize.

Given the absence of safety concerns for foods and feeds from the six-event stack maize and all its subcombinations, the GMO Panel considers that post-market monitoring of these products is not necessary.

The PMEM plan provided by the applicant and the reporting intervals are in line with the intended uses of the six-event stack maize and its subcombinations.

Documentation provided to EFSA

1) Letter from the Competent Authority of Germany received 16 December 2011 concerning a request for authorisation for the placing on the market of maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 (reference EFSA-GMO-DE-2011-103) submitted in accordance with Regulation (EC) No 1829/2003 by Syngenta.
2) Application EFSA-GMO-DE-2011-103 validated by EFSA, 18 August 2014.
3) EFSA stops the clock on 20 August 2014 due to the ongoing risk assessment of application EFSA-GMO-DE-2011-95.
4) EFSA maintains the clock stopped on 7 May 2015 due to the inconclusive opinion of the maize single event 5307 (application EFSA-GMO-DE-2011-95).
5) Receipt of spontaneous information from the applicant, 20 July 2015.
6) Request for supplementary information to the applicant, 21 September 2015.
7) Receipt of supplementary information from the applicant, 29 September 2015.
8) Receipt of supplementary information from the applicant, 16 December 2015.
9) Request for supplementary information to the applicant, 13 March 2018.
10) Request for supplementary information to the applicant, 21 March 2018.
11) Receipt of supplementary information from the applicant, 3 May 2018.
12) Request for supplementary information to the applicant, 4 May 2018.
13) Receipt of supplementary information from the applicant, 25 May 2018.
14) Request for supplementary information to the applicant, 8 June 2018.
15) Receipt of supplementary information from the applicant, 7 June 2018.
16) Request for supplementary information to the applicant, 19 June 2018.
17) Request for supplementary information to the applicant, 18 July 2018.
18) Receipt of supplementary information from the applicant, 6 August 2018.
19) Receipt of supplementary information from the applicant, 29 August 2018.
20) Receipt of supplementary information from the applicant, 17 September 2018.
21) Request for supplementary information to the applicant, 9 October 2018.

22) Request for supplementary information to the applicant, 28 November 2018.
23) Receipt of supplementary information from the applicant, 17 December 2018.
24) Receipt of supplementary information from the applicant, 7 February 2019.

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Abbreviations

ADF	acid detergent fibre
ANOVA	analysis of variance
bp	base pair
Cry	crystal protein
dw	dry weight
ERA	environmental risk assessment
ELISA	enzyme-linked immunosorbent assay
FA	fatty acid
GLP	Good Laboratory Practice
GMO	genetically modified organism
GMO Panel	EFSA Panel on Genetically Modified Organisms
HGT	horizontal gene transfer
IgE	immunoglobulin E
LOQ	limit of quantification
LTP	lipid transfer protein
mEPSPS	mutated 5-enolpyruvylshikimate-3-phosphate synthase
MS	Member States
NDF	neutral detergent fibre
OECD	Organisation for Economic Co-operation and Development
ORF	open-reading frame
PAT	phosphinothricin acetyltransferase
PMEM	post-market environmental monitoring
PMI	phosphomannose isomerise
PPP	plant protection products
SAA	sulfur amino acid
TDF	total dietary fibre
UL	tolerable upper intake level
UTR	untranslated region

Appendix A – Protein expression data

Mean, standard deviation and range of protein levels (μg/g dry weight) from maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21, Bt11, MIR162, MIR604, 1507, 5307 and GA21 unsprayed tissues from field trials performed across three locations in the USA in 2012 (n = 14 or n = 15).[a]

Protein	Event(s)	Leaf (V6)	Leaf (R1)	Root (V6)	Root (R1)	Whole plant (V6)	Whole plant (R1)	Pollen (R1)	Grain (R6)	Grain (senescence)
Cry1Ab	**Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21**	107.0[b] ± 34.8[c] (53.6–175.0)[d]	49.38 ± 20.51 (15.48–81.98)	30.73 ± 8.23 (19.23–41.43)	19.77 ± 3.82 (15.06–26.74)	89.89 ± 21.30 (54.31–120.56)	29.19 ± 10.19 (12.45–47.79)	0.07 ± 0.02 (0.05–0.13)	2.68 ± 0.45 (1.93–3.32)	2.56 ± 0.36 (1.81–2.96)
	Bt11	93.2 ± 29.9 (48.3–128.0)	42.47 ± 14.41 (19.90–63.86)	32.03 ± 7.96 (22.67–45.85)	18.71 ± 1.97 (16.10–22.68)	81.59 ± 20.08 (51.72–113.87)	23.76 ± 8.71 (10.27–36.23)	0.07 ± 0.02 (0.05–0.13)	2.47 ± 0.37 (1.84–3.01)	2.45 ± 0.56 (1.25–3.58)
PAT	**Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21**	5.39 ± 1.55 (2.64–7.91)	6.23 ± 1.06 (4.79–8.26)	0.92 ± 0.41 (0.36–1.54)	0.74 ± 0.12 (0.54–0.95)	3.78 ± 0.67 (2.32–4.97)	3.47 ± 0.85 (2.24–4.60)	0.15 ± 0.05[a] (0.12–0.19)	– (< LOQ)	– (< LOQ)
	Bt11	0.47 ± 0.13 (0.27–0.75)	0.51 ± 0.08 (0.33–0.64)	0.76 ± 0.30 (0.30–1.19)	0.70 ± 0.12 (0.48–0.87)	0.55 ± 0.11 (0.38–0.82)	0.56 ± 0.06 (0.46–0.70)	– (< LOQ)	– (< LOQ)	– (< LOQ)
	1507	4.74 ± 1.36 (2.32–7.76)	5.61 ± 0.96 (3.52–6.96)	0.54 ± 0.26 (0.11–1.0)	0.26 ± 0.07 (0.15–0.38)	3.65 ± 1.25 (1.42–5.79)	2.89 ± 1.08 (1.68–5.28)	– (< LOQ)	– (< LOQ)	– (< LOQ)
Vip3Aa20	**Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21**	134.79 ± 18.60 (108.03–161.56)	145.46 ± 40.70 (90.20–228.15)	92.22 ± 35.36 (53.67–150.70)	48.43 ± 5.67 (39.55–57.76)	156.08 ± 40.40 (64.26–235.51)	124.81 ± 22.70 (85.08–169.36)	112.85 ± 13.50 (90.43–135.15)	99.70 ± 19.01 (75.82–143.22)	90.52 ± 15.81 (69.33–115.87)
	MIR162	168.81 ± 42.80 (109.36–243.83)	134.82 ± 39.10 (70.10–207.68)	82.54 ± 27.01 (44.58–130.06)	45.10 ± 7.30 (32.10–58.20)	151.58 ± 37.60 (68.01–195.90)	138.00 ± 33.60 (91.70–203.0)	115.83 ± 15.60 (82.89–141.51)	101.71 ± 15.0 (80.56–133.83)	91.80 ± 21.61 (40.30–128.0)
PMI[e]	**Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21**	23.69 ± 4.97 (15.94–33.59)	21.59 ± 4.53 (11.13–28.03)	15.22 ± 4.03 (9.18–23.64)	7.46 ± 1.33 (5.25–8.97)	24.37 ± 5.09 (12.43–30.69)	16.61 ± 5.75 (8.55–24.86)	134.15 ± 15.90 (102.18–162.43)	6.38 ± 1.04 (4.67–8.31)	5.66 ± 0.85 (4.70–7.16)
	MIR162	8.01 ± 0.71 (7.13–9.33)	8.79 ± 1.10 (6.93–11.29)	4.47 ± 0.92 (2.85–6.26)	2.23 ± 0.58 (1.45–3.39)	7.66 ± 1.34 (5.36–9.39)	7.44 ± 2.27 (4.09–10.54)	6.74 ± 1.32 (3.91–8.85)	2.10 ± 0.28 (1.78–2.68)	1.91 ± 0.32 (1.54–2.63)
	MIR604	11.35 ± 1.69 (9.58–15.25)	10.35 ± 2.11 (8.02–14.73)	7.59 ± 1.12 (5.97–10.28)	3.94 ± 0.91 (2.76–5.83)	12.18 ± 2.81 (9.28–19.70)	9.22 ± 2.64 (6.24–14.39)	65.89 ± 7.80 (45.99–78.64)	3.14 ± 0.58 (1.96–4.10)	2.94 ± 0.65 (2.27–4.50)
	5307	3.37 ± 0.70 (2.56–4.80)	4.45 ± 0.70 (3.21–6.08)	3.25 ± 0.78 (2.31–5.41)	2.19 ± 0.80 (1.33–3.81)	3.65 ± 0.81 (2.57–5.60)	4.18 ± 0.68 (3.11–5.35)	68.69 ± 7.01 (56.08–80.99)	1.82 ± 0.36 (1.30–2.63)	1.50 ± 0.33 (0.94–2.10)

Protein	Event(s)	Leaf (V6)	Leaf (R1)	Root (V6)	Root (R1)	Whole plant (V6)	Whole plant (R1)	Pollen (R1)	Grain (R6)	Grain (senescence)
mCry3A	**Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21**	13.37 ± 2.14 (9.64–16.78)	10.60 ± 3.38 (4.74–16.78)	13.74 ± 3.01 (9.79–19.79)	11.10 ± 1.39 (9.23–14.29)	11.71 ± 1.83 (8.16–14.34)	7.34 ± 1.54 (4.90–10.01)	0.09 ± 0.03 (0.04–0.15)	0.44 ± 0.24 (0.26–0.97)	0.33 ± 0.10 (0.26–0.64)
	MIR604	15.66 ± 2.49 (11.64–21.05)	13.78 ± 1.89 (10.76–17.11)	17.24 ± 4.35 (10.43–25.64)	12.86 ± 2.32 (8.90–17.43)	13.98 ± 2.88 (8.50–18.47)	10.48 ± 2.43 (6.21–13.83)	0.06 ± 0.02 (0.04–0.10)	0.76 ± 0.30 (0.26–1.32)	0.64 ± 0.21 (0.28–0.99)
Cry1F	**Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21**	26.69 ± 5.39 (17.89–33.98)	20.66 ± 3.32 (14.48–27.66)	10.67 ± 3.73 (5.12–20.26)	7.28 ± 1.30 (5.63–9.50)	20.56 ± 1.88 (16.19–23.11)	13.02 ± 3.34 (7.89 –20.20)	35.08 ± 3.12 (31.86–44.30)	4.92 ± 1.03 (3.64–7.90)	4.42 ± 0.92 (3.38–7.03)
	1507	26.86 ± 4.09 (22.46–34.94)	19.62 ± 3.79 (14.14–26.91)	9.66 ± 2.31 (4.15–12.95)	6.54 ± 1.25 (4.79–8.67)	19.80 ± 2.81 (13.86–24.28)	13.40 ± 2.55 (8.96–17.80)	34.87 ± 5.05 (25.99–42.52)	4.99 ± 0.98 (4.09–7.69)	4.48 ± 0.90 (3.26–5.99)
eCry3.1Ab	**Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21**	104.10 ± 31.70 (66.02–196.15)	43.07 ± 19.70 (9.40–68.82)	26.54 ± 10.95 (9.48–43.68)	7.35 ± 1.89 (3.83–9.83)	125.30 ± 32.10 (68.42–185.74)	32.71 ± 12.25 (11.65–50.92)	0.12 ± 0.03 (0.09–0.16)	4.29 ± 1.21[(a)] (2.10–6.06)	3.38 ± 1.06 (2.27–6.50)
	5307	110.57 ± 20.80 (74.50–151.64)	58.86 ± 10.75 (42.57–80.13)	28.08 ± 14.16 (7.80–57.01)	9.19 ± 2.35 (6.07–13.54)	123 ± 40.60 (73.34–197.0)	53.81 ± 12.65 (31.57–73.64)	0.12 ± 0.03 (0.08–0.16)	6.68 ± 1.72 (4.48–10.09)	4.09 ± 0.96 (2.55–6.03)
mEPSPS	**Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21**	64.64 ± 31.38 (20.32–108.57)	69.09 ± 23.04 (33.58–109.20)	37.70 ± 8.69[(a)] (30.0–51.30)	18.0[(a)]	63.53 ± 19.94 (31.73–96.15)	– (< LOQ)	400.90 ± 64.50 (298.02–500.78)	7.86 ± 0.61[(a)] (7.20–8.70)	5.64 ± 0.08[(a)] (5.58–5.69)
	GA21	61.82 ± 25.07 (26.38–111.81)	81.40 ± 14.23 (59.10–103.0)	36.60 ± 14.57[(a)] (25.80–61.0)	20.90 ± 5.08[(a)] (17.90–26.80)	50.20 ± 14.54 (32.47–74.06)	– (< LOQ)	369.46 ± 50.60 (296.0–469.41)	8.48 ± 1.47[(a)] (6.07–9.97)	6.85[(a)]

(a): Number of samples is n = 14 or n = 15 except for: n = 2 for PAT in pollen of the six-event stack; n = 3 for eCry3.1Ab in pollen of the six-event stack; n = 9 for eCry3.1Ab in pollen of 5307; n = 5 for mEPSPS in root(V6) of the six-event stack and GA21; n = 1 and n = 3 for mEPSPS in root (R1) of the six-event stack and GA21, respectively; n = 5 for mEPSPS in grain(R6) of the six-event stack and GA21; n = 2 and n = 1 for mEPSPS in grain (senescence) of six-event stack and GA21, respectively.
(b): Mean.
(c): Standard deviation.
(d): Range.
(e): PMI levels in maize Bt11 × MIR162 × MIR604 × 1507 × 5307 × GA21 are a sum of two protein variants; one expressed in MIR162 and 5307 and another expressed in MIR604. These two PMI variants differ by two amino acids.
–: Not determined due to all measurements below LOQ.
LOQ: limit of quantification.